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Quantitative TaqMan® real-time PCR assays for gene expression normalisation in feline tissues

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Inhalt

1 Summary

Gene expression analysis is an important tool in contemporary research, with real-time PCR as the method of choice for accurate and fast quantification of mRNA transcription levels. Co-analysis of reference genes is crucial for expression normalization. Since, reference gene expression may vary, e.g., among different species and tissues, their applicability must be tested prior to use in expression studies. The domestic cat is an important study subject in medical research (e.g. animal model for infectious diseases or endocrine disorders) and in veterinary medicine. Little is known about feline reference genes and their application in TaqMan® real-time PCR assays. The aim of the present study was to develop TaqMan® assays for eight potential feline reference genes and to test their applicability for feline samples, including blood, lymphoid, endocrine, and gastrointestinal tissues from clinically healthy cats, and neoplastic tissues. RNA extraction from tissues was optimized for minimal gDNA contamination without use of a DNase treatment. Candidate reference genes included: ABL (v-abl Abelson murine leukemia viral oncogene homolog), ACTB (ß-actin), B2M (ß-2-microglobulin), GUSB (ß-glucuronidase), HMBS (hydroxymethyl-bilane synthase), HPRT (hypoxanthine phosphoribosyltransferase), RPS7 (ribosomal protein S7), YWHAZ (tryptophan 5-monooxygenase activation protein, zeta polypeptide). The assays were tested together with previously developed TaqMan® assays for feline GAPDH

(glyceraldehyde-3-phosphate dehydrogenase) and the universal 18S rRNA gene. The suitability of the candidate genes was assessed using the geNorm and NormFinder programs. A significant difference was found among the expression levels of the ten candidate reference genes ($pKW < 0.001$): the expression levels for the 18S rRNA gene were > 10^6-times higher than those of ABL and HMBS. This will allow matching the expression level of the reference genes with that of the target genes. The presence of pseudogenes was confirmed for four of the eight tested genes. The study confirmed that reference gene expression stability varies considerably among the tested feline tissues. No reference gene was suitable for optimal gene expression normalization in all tissues. For the majority of the tissues, two to four reference genes were found to be a recommendable number of genes for accurate normalization. ACTB, RPS7, and ABL were among the most stable reference genes in the studied tissues, while HPRT, 18S rRNA gene and GAPDH were among the least stable ones. The present study yields essential information on the correct choice of feline reference genes depending on the tissues analyzed.

2 Introduction

The domestic cat is an important study subject not only in veterinary medicine but also in medical research. It plays an essential role as a laboratory model for human infectious, hereditary and endocrine diseases and allows the study of topics such as host-pathogen interactions, defence mechanisms, and development of prophylactic or therapeutic regimens. Of importance in this context is not only the feline immunodeficiency virus (FIV), the single naturally occurring animal model for HIV-AIDS pathogenesis (11, 37) and the feline leukaemia virus (FeLV), an important model for retrovirus and tumor research (33, 38), but also other infectious agents, some of them related to fatal human infections (2, 37). Furthermore, in the genome of the cat, various mutations have been characterised that are associated with genetic diseases, and 280 phenes have been reported, 136 of which could potentially serve as models for human hereditary diseases (http://omia.angis.org.au/). Models under investigation include the glycogen storage disease type IV reported in the Norwegian Forest cat, the only reported animal model for this pathology (16), or the obesity-associated form of diabetes mellitus in the domestic cat that is similar to the type 2 diabetes mellitus in humans (50). For the latter, the domestic cat presents a valuable model for understanding the molecular mechanisms linking obesity to the development of insulin resistance, hypertension, and atherosclerosis (50). The potential of the cat as an animal model and the similarities in genome organisation between humans and felines (35, 36) provide the basis for a wide range of gene expression studies.

Quantitative real-time PCR assays are the method of choice for reliable and fast quantification of transcription levels in gene expression studies, and they are used frequently in many areas of modern research. Real-time PCR provides quantification of input templates over a broad linear range, low

sample consumption, rapid throughput of large sample numbers, and low risk of contamination (19, 21, 30). Accurate normalisation is of fundamental importance to obtaining sound results. Normalisation is usually achieved by simultaneous amplification of reference genes along with the target gene. Several publications emphasise the need for more than one reference gene for exact analysis of transcription levels (39, 40, 46). When selecting reference genes, several critical points should be considered, including a stable, experimentally-independent expression pattern of the candidate gene, the absence of processed pseudogenes, an adequate level of expression, and a lack of potential co-regulation among target and reference genes (22, 41, 43).

For the domestic cat some potential reference genes have been studied using pair-wise correlation analysis (geNorm) (47) and real-time PCR systems based mainly on SYBR Green chemistry (9, 24, 25, 39). The SYBR Green principle has the advantage that it is less costly; however, TaqMan® systems usually have a higher specificity and lead to less non-specific product formation than SYBR Green assays. No systematic study using TaqMan® real-time PCR assays for potential feline reference genes is available. In addition, no comparisons of pair-wise analysis with ANOVA-based methods (NormFinder) (1) have been published, and most assays for feline reference genes were conducted using pathological samples. Data on tissues from healthy cats are largely missing.

Thus, the purpose of the present study was to i) develop and optimise TaqMan® real-time PCR assays for potential feline reference genes and ii) evaluate the suitability of these assays for normalisation in the blood and other tissues from clinically healthy cats and from neoplastic tissues. The earlier tissues were chosen to cover those frequently included in studies investigating infectious diseases and immunological, endocrine, metabolic, and inflammatory disorders. The neoplastic tissues originated from FeLV-infected

cats; the expression of reference genes may differ in neoplastic tissues (22, 26). For stability comparison of the potential reference genes, two programs were used: the ANOVA-based NormFinder and the geNorm, which does pair-wise calculations.

3 Materials and Methods

3.1 Sample collection

All domestic cats included in this study had been in experimental studies officially approved by the veterinary office of the appropriate Swiss Canton. They were kept in groups under optimal ethological conditions. Clinically healthy cats were available from negative control groups, and they were sacrificed for reasons unrelated to this study. Tissue samples were collected upon necropsy from 15 clinically healthy cats (ten neutered males and five intact females). They originated from lymphatic tissues including bone marrow (n = 11), mesenteric lymph node (n = 10), and spleen (n = 10); from the endocrine tissues of the adrenal gland (n = 11), pancreas (n = 13), thyroid (n = 10), and parathyroid (n = 7); from the gastrointestinal tissues of the parotid gland (n = 9), duodenum (n = 10), and ileum (n = 10); and from the brain (n = 13), myocardium (n = 10), kidney (n = 14), and liver (n = 9). The cats ranged in age from 1.25 to 13 years (median age 3.8 years). In addition, EDTA-anticoagulated whole blood samples were collected from 11 specific pathogen-free (SPF) cats (five males at the age of 0.5 years, five neutered males at 6 years, and two spayed females at 14 years). Upon necropsy, neoplastic tissues (n = 12, including tissue from liver (1), spleen (2), kidney (2), mesenteric lymph node (3), ileum (1), and thymus (3) were collected from six FeLV-infected cats (three neutered males, one neutered and two intact females; ages of 3 to 13 years). Five of the cats had been diagnosed with malignant lymphoma; one had leucosis. All tissues were snap-frozen upon collection and stored at -80°C until extraction of nucleic acids.

3.2 Nucleic acid extractions

Tissues (30-35 mg, in duplicate) were homogenised prior to RNA extraction in 350 µl of RLT buffer (Qiagen, Hombrechtikon, Switzerland) containing 3.5 µl ß-mercaptoethanol, together with a 5 mm Ø steel bead (Schieritz & Hauenstein, Arlesheim, Switzerland) in a Mixer Mill MM 300 (Retsch, Haan, Germany). Samples were then processed using the RNeasy Mini Kit (Qiagen) following the manufacturer's recommendations. In a preliminary experiment using selected samples (n = 4), the effect of a digestion step on the RNA binding silica gel membrane of the spin column, performed according to the manufacturer's instructions with RNase-free DNase, was assessed (on-column DNase treatment). In addition, for bone marrow, lymph node, spleen, and thyroid samples, the RNeasy Plus Mini Kit (Qiagen) with genomic DNA (gDNA) Eliminator spin columns and RLT plus buffer was applied according to the manufacturer's recommendations. The presence of contaminating gDNA was assessed using GAPDH quantitative reverse transcriptase (RT-) PCR with a minus-reverse transcription control with Reverse Transcriptase qPCR Mastermix (Eurogentec, Seraing, Belgium). RNA was extracted from 1 ml of blood within 60 minutes of collection using the QIAamp Blood Mini Kit (Qiagen) and stored at -80°C until further use. gDNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen). For all RNA and DNA extractions, negative controls were prepared with each batch.

3.3 First-strand cDNA synthesis

The RNA yield and the ratio of absorbance at 260 nm to 280 nm (A_{260}/A_{280} ratio) were measured using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Witec, Littau, Switzerland). Samples containing < 10 ng/µl of RNA were excluded from the study. First-strand cDNA was

synthesised in quadruplicate using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Rotkreuz, Switzerland) according to the manufacturer's instructions. The amount of input RNA in each reaction was calculated to be 2 µg. The cDNA GAPDH copy number / RNA GAPDH copy number ratio was calculated as a measure of the efficiency of the cDNA synthesis; this ratio was used to normalise the reference gene copy numbers as assessed by quantitative real-time PCR.

3.4 Development of real-time PCR assays for feline reference genes

Using Primer Express™ software (versions 2 and 3, Applied Biosystems), primers and TaqMan® probes were designed for eight potential reference genes: ABL, ACTB, B2M, GUSB, HMBS, HPRT, RPS7, and YWHAZ (essential gene-specific data are given in Table 1). The sequences and information on gene organisation were retrieved from Ensembl (http://www.ensembl.org/index.html), GenBank (http://www.ncbi.nlm.nih.gov) and the Genome Annotation Resource Fields (GARFIELD) (http://lgd.abcc.ncifcrf.gov) (41). All systems were designed so that the predicted amplicons would span exon-exon boundaries (Table 2). The eight primer pairs (Microsynth, Balgach, Switzerland) were tested for amplification of the appropriate length amplicon using 5 µl of cDNA in a total volume of 25 µl per reaction on a Rotor-Gene6000 real-time rotary analyser (Corbett, Mortlake, Australia) using the TaqMan® Fast Universal PCR Master Mix (Applied Biosystems). Thermocycling conditions consisted of an initial denaturation of 20 s at 95°C, followed by 45 cycles of 95°C for 3 s and 60°C for 45 s. The PCR products were analysed by gel electrophoresis on 3% agarose gels and stained with ethidium bromide, and bands were visualised using the Chemigenius2 BioImaging System (Syngene, Cambridge, UK).

In order to test for potential amplification of pseudogenes or of gDNA, the eight primer pairs were also assayed with gDNA under the same conditions. Moreover, the possible presence of pseudogenes for the eight assays was assessed using the Ensembl Genome Browser. In all PCR assays, water was used as a negative control.

3.5 Optimisation of quantitative real-time PCR assays

After the primers had been tested for correct amplification of the estimated amplicon length, the eight newly designed real-time TaqMan® PCR systems were optimised using cDNA and a 3 x 3 primer matrix with 50, 300, and 900 nM end concentrations. Each of the nine conditions was run in quadruplicate under the conditions described above. Moreover, using the best primer concentration, five different probe (Microsynth) end concentrations (50, 100, 150, 200, and 250 nM) were tested for optimal performance. The optimised assays were tested together with a feline GAPDH TaqMan® real-time PCR assay developed previously in our laboratory (28) and a universal 18S rRNA gene (Applied Biosystems).

3.6 Production of DNA standards for absolute quantification

cDNA synthesised from feline tissue samples was used to generate standard templates for absolute quantification of ABL, ACTB, B2M, GUSB, HMBS, RPS7, and YWHAZ. The corresponding sequences were amplified using primers enclosing the TaqMan® real-time PCR sequences under conditions described (Table 3) (49). The gel purified amplification products (Gen Elute PCR Clean-Up Kit, Sigma-Aldrich, Buchs, Switzerland) were subjected to a 3' A-tailing reaction (Sigma) and ligated into the TOPO TA cloning vector pCRII (Invitrogen, Basel, Switzerland), selected by Ampicillin resistance, followed by

sequencing (Microsynth). Plasmids were linearised by restriction digestion with *BamH*I (Promega, Wallisellen, Switzerland), *Spe*I (New England BioLabs, Berverly, MA, USA), or *Kpn*I (Roche, Rotkreuz, Switzerland) and then gel purified. The copy numbers were calculated based on spectrophotometric analysis (NanoDrop ND-1000). Carrier salmon sperm DNA (Invitrogen) at a concentration of 30 µg/ml was used for the tenfold serial dilutions of the standard templates, and aliquots of the dilutions were stored at -20°C until use. For the GAPDH assay, the DNA standard described previously (28) was used. For the HPRT and 18S rRNA assays, cDNA from kidney tissue of a clinically healthy cat was diluted tenfold in carrier salmon sperm DNA and in nuclease free water, respectively, to produce an arbitrary standard. The copy numbers of the latter samples were estimated by matching the resulting Ct values with those of the feline GAPDH standard.

3.7 Efficiency, sensitivity, linear range and precision of the real-time PCR assays

The efficiency of the newly designed assays was calculated as described (23) using the following equation: $E = (10^{(-1/slope)})-1$. The sensitivity of the seven new systems (for which DNA standards had been produced) was determined by an endpoint dilution experiment: ten replicates of the dilutions containing 10^2, 10^1, and 10^0 standard template copies per reaction, respectively, were tested. The sensitivity of the assay is given by the dilution in which at least seven of 10 replicates are still positive (31). The linear range of amplification and the precision of all newly developed TaqMan® real-time PCR assays were determined using tenfold serial dilutions of the plasmid or arbitrary standards. For the precision analysis, the dilutions were chosen according to the ranges of Ct values that were characteristic for the expression levels of the particular

reference genes in the tissues. Intra-run (n = 10) and between-run (n = 5) analytical performances of the PCR measurements were determined using these control materials.

3.8 Data evaluation statistics

For stability comparison of candidate reference genes, the Microsoft Excel Add-in NormFinder (1) was applied. Comparisons were made with calculations performed using the geNorm version 3.4 (48). The NormFinder uses an ANOVA-based model (1), while the geNorm calculates the stability using a pairwise comparison model (48). In addition, the geNorm ranks candidate reference genes according to the average expression stability, *M* (48). Genes with the lowest *M* values have the most stable expression; a cut-off of 1.5 was proposed, above which the variation is assumed to be too high for accurate normalisation (48). Moreover, the optimal number of reference genes required for accurate normalisation was estimated using the geNorm. To this end, the pairwise variation $V_{n/n+1}$ between sequential normalisation factors containing an increasing number of reference genes was calculated. If $V_{n/n+1} < 0.15$, the recommended number of reference genes is given by n; the inclusion of an additional reference gene is not required (48).

Statistical analyses were performed with GraphPad Prism for Windows, Version 4.03 (GraphPad software, San Diego, CA). Expression levels of individual genes in different tissues were tested for statistical differences among several groups using the non-parametric Kruskal-Wallis test (pKW) and the Dunn's Multiple Comparison Test (pD). The expression levels of different genes in individual samples were tested for statistical differences between two groups using the non-parametric Wilcoxon signed rank test for paired samples (pW) and among several groups using the non-parametric Friedman test for

paired samples (pF) and the Dunn's Multiple Comparison Test (pD). P-values < 0.05 were considered significantly different.

Results

3.9 RNA extractions and gDNA contamination

RNA extractions from blood using the QIAamp Blood Mini Kit and from tissues using the RNeasy Mini Kit yielded RNA with a low level of contaminating gDNA (<1%), with the exceptions of bone marrow, lymph node, spleen, and thyroid. When RNA extraction was performed for these four tissues using the RNeasy Plus Mini Kit, contaminating gDNA levels were < 1%. These additionally processed RNA samples were used for analysis of these four tissues. No on-column DNase treatment was used in the main experiment because the loss of RNA due to DNase treatment was > 90%, as determined in a preliminary experiment (data not shown). RNA purity was estimated from A_{260}/A_{280} ratio; this ratio ranged from 1.7 to 2.1.

3.10 Evaluation of the primer pairs

When the primers were tested in a conventional PCR with cDNA, all assays yielded PCR products of the predicted size (Table 2). The primers were then assessed using the same procedure and gDNA to test for the amplification of gDNA and the possible presence of pseudogenes. Bands of the size of the cDNA were found for ACTB, HPRT, RPS7, and YWHAZ, indicating the presence of processed pseudogenes for these genes, but not for ABL, B2M, HMBS, and GUSB (Table 2). This was consistent with the results retrieved from Ensembl. In addition, for ACTB, GUSB, and HMBS, PCR products presumably of the size of the gDNA, including the introns, were detected

(Table 2). For ACTB, it was shorter (~400 bp) than calculated from an alignment of the human with the feline sequence (568 bp, Table 2).

3.11 Evaluation and optimisation of the newly developed real-time PCR assays

Primer and probe concentrations for the eight newly designed TaqMan® real-time PCR assays were optimised using cDNA (for final concentrations see Table 4). When the real-time TaqMan® PCR assays were tested using gDNA instead of cDNA, specific amplification was found for ACTB, HPRT, RPS7, and YWHAZ, confirming the presence of pseudogenes (Table 2). In addition, amplification was detected for GUSB (Table 2).

3.12 Efficiency, sensitivity, linear range and precision of the real-time PCR assays

The amplification efficiencies of the eight newly designed assays and the feline GAPDH real-time PCR were ≥ 96%. The lower detection limit of the assays for ABL, ACTB, B2M, GUSB, RPS7, and YWHAZ was equal to one copy of target standard plasmid per reaction in an endpoint dilution experiment (7 to 10 out of 10 reactions positive). For HMBS the lower limit of detection was found to be < 100 copies per reaction (10 out of 10 reactions positive). For all newly developed TaqMan® real-time PCR assays, we observed linearity of the assay over a $\geq 10^8$-fold range. The coefficients of variation ranged from 0.44% (B2M) to 1.18% (YWHAZ) for the intra-run precision analysis and from 0.49% (ACTB) to 2.15% (HMBS) for the between-run analysis.

3.13 Expression levels of candidate reference genes

Transcription of the ten candidate reference genes was detectable above background in all tissue and blood samples from all cats tested. The potential reference genes were classified into three groups according to their transcription levels (all healthy tissues and blood samples were included in the analysis; Figure 1a). The difference in median expression levels was found to be 10^6 between the most abundant and least abundant transcripts: 18S rRNA showed a high transcription level (median copy number/reaction ~10^9); ACTB, GAPDH, B2M, HPRT, and RPS7 were found to have intermediate transcription levels (median copy number/reaction $2.6x10^4$ to $9.3x10^4$), and ABL, GUSB, and HMBS had low transcription levels (median copy number/reaction $0.5x10^3$ to $1.9x10^3$; Figure 1a). YWHAZ had a transcription level between the intermediate and low levels (median copy number/reaction $6.9x10^3$). Individual candidate reference genes had different expression levels across all studied tissues; the transcription levels differed significantly among all different reference genes when all tissues were included in the analysis ($pF < 0.0001$; $pD < 0.05$), with the exceptions of ACTB, GAPDH and B2M; GUSB and HMBS; and HPRT and RPS7 (Figure 1a). The latter three groups of reference genes did not have significantly different transcription levels ($pD > 0.05$; Figure 1a). A reference gene transcription level pattern similar to that seen in all tissues combined was found when individual tissues were examined (for a representative example, see Figure 1b), with some particular exceptions. In the bone marrow samples HMBS transcription was significantly higher than GUSB transcription ($pW = 0.0010$; Figure 1c), in the myocardium and brain samples GAPDH transcription was significantly higher than B2M transcription ($pW = 0.0020$; Figure 1d and 1e), in the blood B2M and ACTB transcription levels were higher than GAPDH transcription ($pW = 0.0039$; Figure 1f), and in

the liver YWHAZ was significantly lower than RPS7 and HPRT (pW = 0.0039, data not shown).

When expression levels of the individual potential reference genes were analysed among different tissues some significant differences were found. The most prominent were the following: GAPDH was significantly higher in the myocardium, brain, and blood samples than in most of the other tested tissues (pKW < 0.0001; pD < 0.001 for 11 of the other tested tissues; Figure 2a); ABL was significantly lower in the bone marrow samples than in the majority of the other tested tissues (pKW < 0.0001; pD < 0.001 for eight of the other tested tissues; Figure 2b), B2M was higher in the blood samples (pKW < 0.0001; pD < 0.001 for twelve of the other tested tissues; Figure 2c), and YWHAZ was higher in the blood and brain samples than in the majority of the other tissue samples (pKW < 0.0001; pD < 0.001 for 11 and 12, respectively, of the other tested tissues; Figure 2d). No particular differences in expression levels were observed when the neoplastic tissues were compared to the healthy tissues (pW > 0.05 for all genes tested).

3.14 Expression stability of candidate reference genes in different tissues

The stability of reference gene expression was estimated based on the calculations of the geNorm and NormFinder software, and the rank order given by the two programs differed significantly (Table 5; for details see also Appendix). However, some agreement was found between the NormFinder results and the results according to the *M* values of the geNorm program (Table 5); for three tissues (bone marrow, duodenum, and kidney) the rank order was identical. The two genes that ranked best were identical with both methods (manual *M* value ranking and NormFinder) for the majority of the

tissues except for lymph node, parotid gland, liver, and blood samples. All further stability analyses were made using the NormFinder results.

The stability of the genes varied considerably depending on the tissues tested. When tissues were analysed individually, no single gene was found among the three best-ranked genes in all of the tested tissues. In 11 of the 14 healthy tissues, RPS7 ranked among the three most stable genes, followed by ACTB in eight and ABL in six out of 14 tissues (Table 5). Somewhat less stable were GUSB and YWHAZ; they were among the most stable genes in five of the 14 tissues used. GAPDH was among the three most stable genes in the NormFinder ranking in four out of 14 tissues. HMBS, B2M, HPRT, and 18S rRNA were found to be the least stable, HMBS with two, B2M and HPRT with one, and 18S rRNA with no rankings among the most stable genes in the 14 tissues tested. When all 14 healthy tissues were included at once in the analysis (Table 5), RPS7, GUSB, and YWHAZ ranked as the best three reference genes, while ABL and ACTB ranked fourth and fifth. Some particular differences were found in certain healthy tissues; RPS7, which ranked first overall, was found to be less stable in the ileum, lymph node, and thyroid samples. Moreover, ACTB ranked last in the pancreas samples (Table 5). In the blood samples, GAPDH and B2M ranked better than in other tissues, but RPS7 ranked only second to last (Table 5). Remarkably, in the neoplastic tissues the two most stable genes were identical to those in all the healthy tissues (RPS7 and ACTB). ABL, GUSB, and YWHAZ were found to be less stable than in the healthy tissues (Table 5).

3.15 Number of reference genes for optimal normalisation

The optimal number of reference genes for normalisation was calculated using the geNorm program. The number of recommended reference genes for

optimal normalisation varied considerably depending on the tissue being tested. For brain, myocardium, lymph node, and adrenal gland the pairwise variation $V_{2/3}$ was ≤ the proposed cut-off of 0.15 (Appendix); therefore, two reference genes should be sufficient for accurate normalisation in those tissues. Similarly, for parathyroid, parotid gland, liver and kidney, three; for thyroid, four; for spleen and neoplastic tissues, five; and for blood, seven reference genes were found to be necessary for accurate normalisation according to the geNorm program (Appendix). For four tissues the pairwise variation *V* always exceeded the cut-off of 0.15. The recommended number of reference genes for these four tissues (lowest *V* value) was: five for the pancreas, six for ileum and bone marrow, and seven for the duodenum (Appendix). For all tissues combined, the optimal number of reference genes that would have been necessary for normalisation exceeded ten using the reference genes tested in this study. For the three tissue groups, endocrine, lymphatic, and gastro-intestinal tissues, the *V* value always exceeded the cut-off of 0.15; the recommended number of reference genes for these tissue groups (lowest *V* value) was found to be six (Appendix).

3.16 Normalisation

When tissues were analysed individually, a normalisation factor was calculated for all tissues with the exception of the pancreas. For the latter, all *M* values calculated by geNorm exceeded the proposed cut-off value of 1.5, and no normalisation factors based on the geometric mean of multiple reference genes could be computed (Appendix 1). When tissue groups were analysed, a normalisation factor was calculated for the endocrine and gastrointestinal tissues but not for the lymphatic group. No normalisation factor was calculated for all tissues combined.

4 Discussion

The present study is the first to develop and evaluate real-time TaqMan® assays for the quantification of a series of potential feline reference genes. The assays were applied using feline peripheral blood and tissue samples from healthy cats. The latter were chosen based on their applicability in research areas such as the investigation of immune functions or metabolism, characterisation of infections, inflammatory reactions, or excretion patterns of pathogens. In this regard, the selected tissue categories also met the claim of suitability regarding their potential use in animal models for several human diseases. The expression stability of potential reference genes was analysed and compared for the feline species for the first time using pair-wise and ANOVA-based analyses. Remarkable differences concerning gene expression stability were found among the different tissues using the different analysis methods.

We selected ten commonly used mammalian reference genes featuring a broad range of cellular functions (Table 1) in order to reduce the risk of co-regulation among the target and reference genes. Moreover, recently it was reported that nine of the ten genes included in this study, i.e., ACTB, ABL, B2M, GAPDH, GUSB, HMBS, HPRT, YWHAZ, and the 18S rRNA gene, are assumed not to be co-regulated (20, 32, 48).

When selecting potential reference genes, another issue that needs to be considered is the presence of processed pseudogenes, which are known to hamper data interpretation in mRNA transcription analysis (17, 51). We therefore included candidate reference genes that are known to lack pseudogenes in humans, i.e., ABL, B2M, GUSB, and HMBS (29, 51). When we tested these four genes in felines, we confirmed that they also lack pseudogenes in the cat (at least for the sequences included in our assays).

For the other four newly developed assays (ACTB, HPRT, RPS7, and YWHAZ), we demonstrated the presence of processed pseudogenes within the sequences targeted by our assays. For feline GAPDH the presence of one copy of a pseudogene had been reported earlier (34). To minimise co-amplification of pseudogenes, gDNA contamination of the assayed RNA must be controlled. In the present study, gDNA contamination was reduced to a minimum (< 1% of the RNA) by choosing the optimal RNA extraction method for each tissue. The inclusion of a DNase digestion step was omitted because we found it to significantly reduce the RNA yield (> 90% loss). The absence of a DNase treatment and thus the presence of sufficient amounts of RNA might explain why we always had PCR signals clearly above background for all samples in the present study, in contrast to some other reports in which HPRT, ABL, or HMBS could not (reliably) be detected (42, 50).

Another method for reducing the possible interference of gDNA - including conventional but not processed pseudogenes - with the RNA under investigation in gene expression studies is the positioning of the real-time PCR target on exon-exon junctions. In the present study, all eight newly developed assays were designed to span such a junction. The assays for those four potential reference genes lacking processed pseudogenes did not amplify gDNA, with the exception of GUSB. For the latter, the amplicon size from gDNA was only 532 bp due to a short intron and the primer design software had positioned the exon-exon junction only three nucleotides before the 3’ end of the probe (Table 2).

The expression level of an ideal reference gene should not undergo tissue-specific and experimental-dependent variation and should be similar to that of the target genes (7, 22, 42, 44, 46, 48). We measured the RNA transcription levels of the ten selected candidate reference genes in blood samples and 15 different tissues from cats. While the expression levels among the reference

genes differed considerably, most of them were within a range that could be used for proper normalisation of many target genes. Only the 18S rRNA gene expression level was found to be impracticably high compared to that of most target genes. Significant differences were found when the expression levels of the potential reference genes were analysed in different tissues. GAPDH expression was higher in brain, myocardium, and blood samples; this is consistent with results reported for human tissues (3, 48). Tissue-specific expression differences could also be detected for B2M, where expression was significantly higher in blood. This has also been demonstrated for human leukocytes, where a significantly higher expression level was found (48).

We then calculated the expression stability of the tested reference genes using the NormFinder and compared it to that calculated by the geNorm software, as well as to a stability ranking performed manually using the *M* values allocated to the individual genes by the geNorm program. The reference gene rankings automatically produced by the two programs were remarkably different. Better agreement was found between the ranking provided by the NormFinder and the manual ranking according to the *M* values of the geNorm. Consequently, and intriguingly, differences were observed in the automatically calculated geNorm gene ranking and the ranking performed manually using the *M* values of the geNorm program. To our knowledge this has not been observed previously.

In general, when comparing results of the NormFinder and the geNorm, the underlying mathematical models need to be considered. The pairwise comparison model of the geNorm program provides a combination of two genes whose expressions are most correlated in the tested sample by stepwise exclusion of the genes with the highest *M* values (48). The stability measure *M* calculated by the program is defined as the pairwise variation between a gene and all the other genes (48). We primarily assessed the

ranking provided by the NormFinder program. In the geNorm program co-regulated genes that show a tendency towards similar expression profiles may become highly ranked independent of their expression stabilities (1). On the other hand, the ANOVA-based model of the NormFinder program selects the highest ranked gene based on the highest expression stability due to minimal estimated intra- and intergroup variation (1). This approach is not significantly affected by co-regulation of candidate reference genes (1). Although we had aimed to avoid including reference genes that are co-regulated (see above), the differences found between the results of the two programs using different mathematical models might indicate that some of the genes were still co-regulated.

Studies of the validation of reference gene expression have been performed for different mammalian species including humans (4, 29, 42, 48), companion animals (6, 12, 39, 40), farm animals (13, 14, 18, 43), and horses (5, 8). No general best reference gene has been found for all species. Moreover, our results demonstrated that in the cat the expression stability of the ten investigated reference genes differed significantly depending on the tested tissues. No single gene was found to be suitable for accurate normalisation in all investigated tissues within one species. Our findings are consistent with the results reported from other mammalian species (7, 27, 42, 46) and highlight the need for proper validation of reference genes in the respective tissues preceding any experimental set-up. It has been demonstrated that normalisation of data sets with different reference genes, such as GAPDH and ACTB, may influence the outcome of the study; the expression profiles of target genes were markedly influenced and statistically significant differences between study groups were present or absent depending on the choice of the reference genes (10, 15, 45).

In the present study, in the majority of the examined healthy tissues, RPS7, ACTB, and ABL were among the most stably expressed genes. When all healthy tissues were combined for analysis, the three most stable genes were found to be RPS7, GUSB, and YWHAZ. These results are only partially in agreement with those of a recent validation of feline reference genes using SYBR Green real-time PCR assays and pairwise analysis (39). In the latter study, RPS7 was also found as the most stable gene in six tissues under investigation; YWHAZ ranked fourth (after two ribosomal protein genes not included in the present study), and GUSB was found among the least stable genes; ACTB and ABL were not examined (39). Three feline tissues were included in both the previous (39) and the present study. While for the kidney at least some agreement was found in the gene ranking (RPS7 and YWHAZ were among the stable ones; HPRT and GAPDH were rather unstable), the results for liver and myocardium differed significantly. The observed differences could be (apart from the differences in the applied methods) due to the fact that while we examined tissues from clinically healthy cats, Penning and co-workers (39) studied tissues from sick cats. They included liver, heart, and kidney samples from cats with hepatocellular carcinoma, hepatitis, cardiac hypertrophy, chronic kidney failure, and kidney tumours (39). Gene expression studies frequently examine pathological tissues and compare them with healthy tissues. The present results emphasise the need to include samples from healthy tissues in the evaluation of suitable reference genes for correct normalisation and comparison of healthy to pathological tissues. We suggest choosing the reference gene according to its suitability in the healthy tissue of the particular species (e.g., the present study of the cat) and then evaluating it in the pathological tissues of interest in a preliminary experiment. Furthermore, the number of samples under evaluation might influence the study outcomes:

we intended to include 10 samples per tissue, while in the previous study, for the majority of the tissues, fewer samples were available (39).

Some particular differences in the reference gene stability were observed in the present study. Although B2M in general was not a very stable reference gene, it ranked second to fourth in stability in the blood samples. This would confirm a finding in human blood cells, where B2M seemed a good choice for normalisation in leukocytes, while it was one of the least stable genes in tissues (48). For the neoplastic tissues, only a limited number of samples from FeLV-infected cats with malignant lymphoma and leucosis were available, and they originated from different tissues. Nonetheless, it is remarkable to note that the expression levels of the chosen reference genes did not differ significantly from those in the healthy tissues. In addition, the two most stable genes in the neoplastic tissues were identical to those in the investigated healthy tissues, although overall some differences were found in the stability ranking of the potential reference genes. Thus, two genes (RPS7 and ACTB) could serve as a good first choice to be tested in these neoplastic tissues as well in future studies.

We calculated the minimum number of reference genes necessary for accurate normalisation using the geNorm program (48). Applying the proposed cut-off value of 0.15 for the pairwise variation $V_{n/n+1}$ (48), two to three reference genes were required for accurate normalisation in most tissues. Moreover, for all tissues except the pancreas, the lymphatic tissue group, and all tissues combined, a normalisation factor was calculated. According to Vandesompele and co-workers, using the three best reference genes is a valid normalisation strategy in most cases; it results in much more accurate and reliable normalisation than the use of only one reference gene does (48). In addition, practical and financial considerations may support the use of a limited number of reference genes. This strategy is supported by the finding that no significant

effect on the relative quantity of the target gene expression was demonstrated when using the combination of the two best genes compared to using five of the six most stable genes (32).

From our results, we recommend consulting the literature for appropriate reference genes according to the tissues and species under investigation. The reference genes should be examined in healthy individuals. If the corresponding data is not available, preliminary experiments for the identification of optimal reference genes are necessary. If this is impossible, we recommend using RPS7 as the primary choice for a reference gene in feline tissues (with tissue-specific limitations documented in the present study); results should be confirmed using other reference genes, such as ACTB, ABL, and/or YWHAZ.

In this study, we investigated for the first time the most reliable feline reference genes for normalisation of gene expression data in blood samples and 15 different tissues, including endocrine, lymphatic, and gastrointestinal tissues, using real-time TaqMan® PCR assays and pair-wise and ANOVA-based analyses. Our results indicated that stability of the reference genes varies among the different tissues, and no gene was found among the most stable ones for all the tissues under investigation. Moreover, significant differences were found using either pair-wise or ANOVA-based analysis approaches. The three most stable genes were found to be RPS7, ACTB, and ABL, while B2M, HPRT, and the 18S rRNA gene were the least stable ones. The frequently used GAPDH had an acceptable stability in only a few tissues. These data emphasise the need for proper validation of candidate reference genes in the respective tissues and species in healthy individuals preceding the initiation of any experimental gene expression study.

5 Figures and Tables

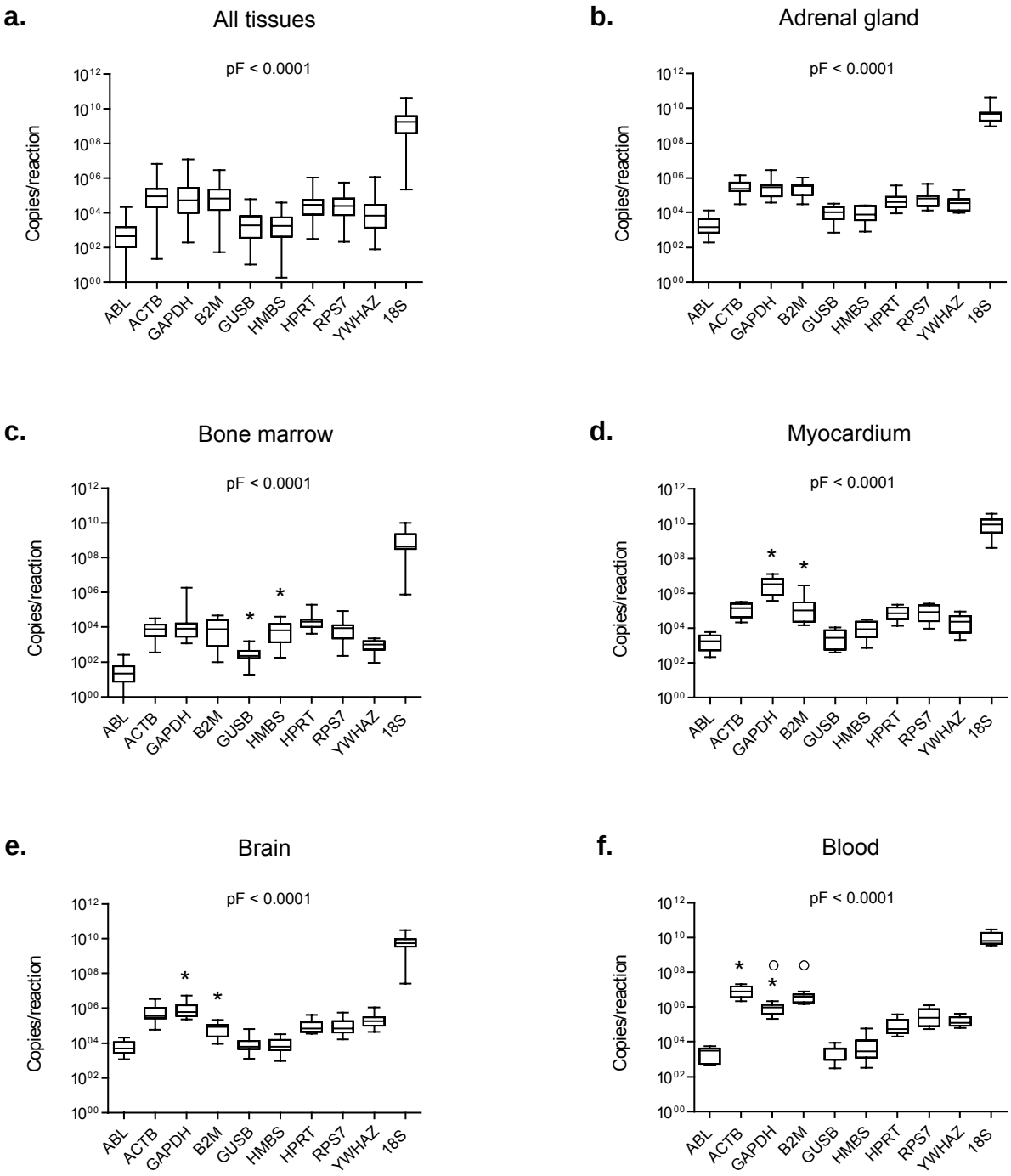

FIGURE 1. Expression levels of candidate reference genes in different tissues. a) All healthy tissues combined, b) adrenal gland, c) bone marrow, d) myocardium, e) brain, and f) blood samples. Values are given as copy numbers per PCR. For the 18S rRNA gene and HPRT the copy numbers were calculated using an arbitrary standard (see M&M). Data are shown as boxplots. Boxes extend from the 25th to the 75th percentile; a horizontal line represents the median, and the error bars extend down to the smallest and up to the largest value. Expression levels were analyzed for statistical differences using the Friedman test (pF-values as indicated in the figure). Significant differences between two particular genes were analyzed by the Wilcoxon test for paired samples (asterisks and circles, respectively, mark statistically significant differences between two genes; $pW \leq 0.0039$).

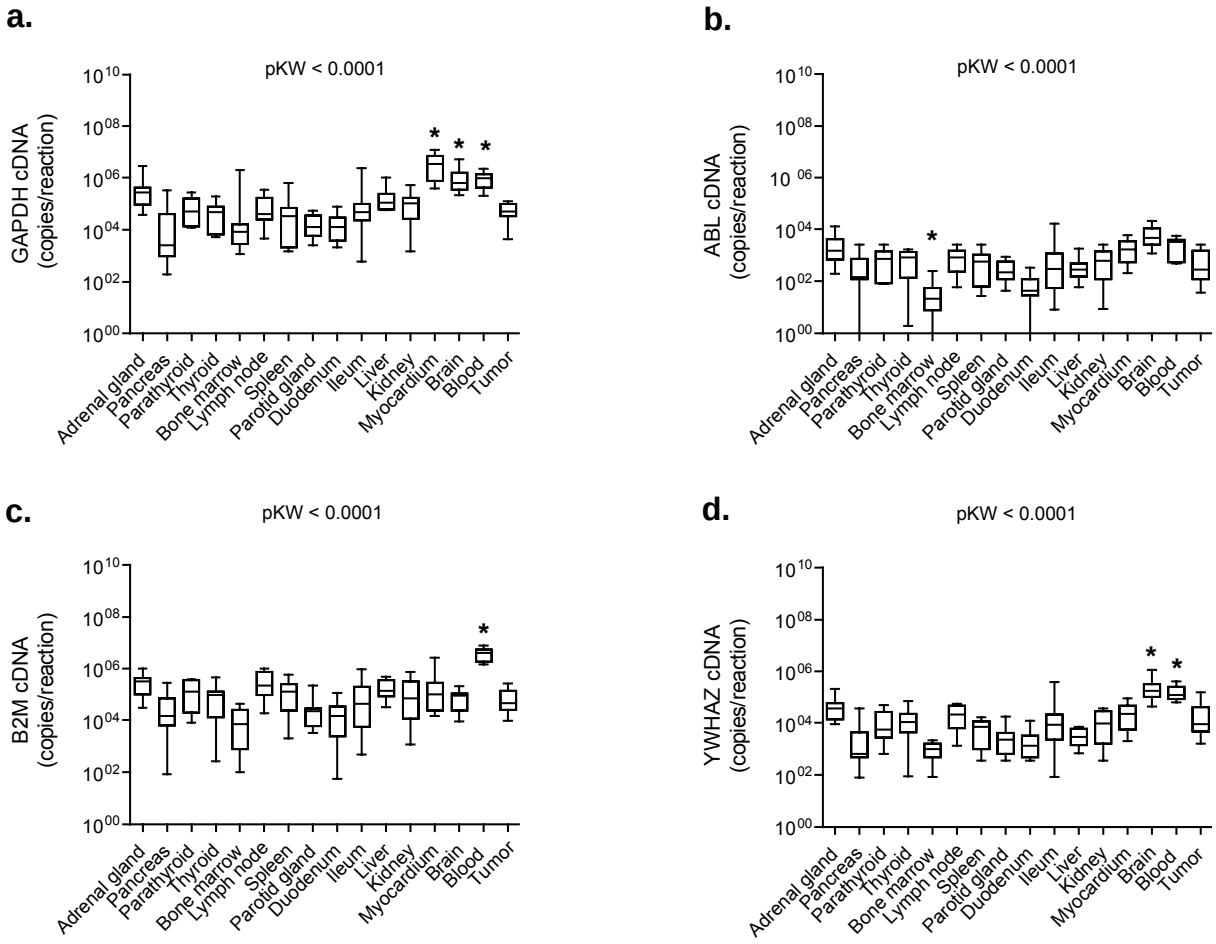

FIGURE 2. Expression levels of selected reference genes in individual tissues. a) GAPDH, b) ABL, c) B2M, and d) YWHAZ. Values are given as copy numbers per PCR. Data are shown as boxplots. Boxes extend from the 25th to the 75th percentile; a horizontal line represents the median, and the error bars extend down to the smallest and up to the largest value. Expression levels were tested for statistical differences by the Kruskal-Wallis test (pKW-values as indicated in the figure) and the Dunn's post test (asterisks indicate statistically significant differences; pD < 0.001).

TABLE 1. Specifications of the tested potential feline reference genes.

Gene	Name	Function	Accession Number
ABL	v-abl Abelson murine leukaemia viral oncogene homolog	Protein kinase; regulation of cell cycle, mismatch repair, DNA damage response	ENSFCAT00000005306[1]
ACTB	ß-actin	Cytoskeletal structural protein	AB051104.1[2]
B2M	ß-2-microglobulin	Major histocompatibility complex antigen class I receptor activity	NM_001009876[2]
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Glycolytic enzyme	AF097177[2]
GUSB	ß-glucuronidase	Glycoside hydrolase (carbohydrate metabolism)	NM_001009310[2]
HMBS	Hydroxymethyl-bilane synthase *Alias*: Porphobilinogen deaminase (PBGD)	Heme synthesis, porphyrin metabolism	ENSFCAG00000001160[1]
HPRT	Hypoxanthine phosphoribosyltransferase	Purine synthesis in salvage pathway	EF453697[2]
RPS7	Ribosomal protein S7	Ribosomal protein	NM_001009832[2]
YWHAZ	Tyrosine 3-monooxygenase *Alias:* tryptophan 5-monooxygenase activation protein, zeta polypeptide *Alias:* Phospholipase A2	Mediator of signal transduction	EF458621[2]
18S rRNA		Ribosomal RNA	X03205[2]

[1] Ensembl, [2] GenBank

TABLE 2. Details of Taqman® real-time PCR assays.

Gene	Oligo	Sequence	Amplicon size (bp)	Genomic position (exon)	Genomic (bp)	Detection gDNA	Pseudo -gene
ABL	Forward	TGTGGCGAGTGGTGATAATACAC	83	2	~12,000 [7]	No	No
	Probe	CAGCATCACTAAAGGTGAAAAGCTACGAGTCCTT [2]		2/3			
	Reverse	TCCACTCACCATTCTGGTTGTAA		3			
ACTB	Forward	CAACCGTGAGAAGATGACTCAGA	127	3/4	568 [8]	(Yes) [11]	Yes
	Probe	TCTCTGTACGCTTCTGGCCGCACC [3]		4			
	Reverse	CCCAGAGTCCATGACAATACCA		4			
B2M	Forward	CGCGTTTTGTGGTCTTGGT	84	1	3,270 [7]	No	No
	Probe	CGGACTGCTCTATCTGTCCCACCTGGA [2]		1			
	Reverse	AAACCTGAACCTTTGGAGAATGC		1/2			
GAPDH	Forward	GCCATCAATGACCCCTTCAT	82	1	NA	(Yes) [11,12]	Yes
	Probe	CTCAACTACATGGTCTACATGTTCCAGTATGATTCCA [4]		1/2			
	Reverse	GCCGTGGAATTTGCCGT		2			
GUSB	Forward	CTACATCGATGACATCACCATCAG	80	4	532 [9]	Yes	No
	Probe	ACCAGCGTGAACCAAGACACTGGGC [3]		4/5			
	Reverse	CGCCTTCAACAAAAATCTGGTAA		5			
HMBS	Forward	TGGCAGTGCTGAAAGCCTTA	94	3	554 [7]	No	No
	Probe	TTGAAATCGTTGCTATGTCCACCACAGG [2]		3/4			
	Reverse	TTAGAGAGCGCAGTATCAAGAATCTT		4			
HPRT	Forward	AACTGGAAAGAATGTCTTGATTGTTG	100	4/5	>90,000 [7]	(Yes) [11]	Yes
	Probe	CACTGGCAAAACAATGCAAACCTTGCTTT [3]		6			
	Reverse	GACCATCTTTGGATTATACTGCTTGA		6			
RPS7	Forward	GTCCCAGAAGCCGCACTTT	74	4/5	~2,200 [10]	(Yes) [11]	Yes
	Probe	CGCCGTGCACGACGCGA [5]		5			
	Reverse	CACAATCTCGCTCGGGAAAA		5			
YWHAZ	Forward	ACAAAGACAGCACGCTAATAATGC	83	4	2,878 [7]	(Yes) [11]	Yes
	Probe	ATTACTGAGAGACAACTTGACATTGTGGACATC [3]		4/5			
	Reverse	CTTCAGCTTCATCTCCTTGGGTAT		5			
18S rRNA [1]	Forward	CGGCTACCACATCCAAGGAA	NA	NA	NA	NT	NT
	Probe	TGCTGGCACCAGACTTGCCCTC [6]		NA			
	Reverse	GCTGGAATTACCGCGGCT		NA			

[1] TaqMan® Gene Expression Assay (Applied Biosystems); [2] 5' FAM/3' BHQ-1; [3] 5' Yakima Yellow/3' BHQ-1; [4] 5' FAM/3' TAMRA; [5] 5' TET/3' TAMRA; [6] 5' VIC/3' MGB; [7] Ensembl; [8] According to alignments of human and feline sequences retrieved from PubMed; [9] According to the Genome Annotation Resource Fields (GARFIELD) (http://lgd.abcc.ncifcrf.gov) (41); [10] According to Penning et al. (39); [11] Amplification of processed pseudogene; [12] According to Leutenegger et al. (28); NA = Not available; NT = Not tested

TABLE 3. Primers used for production of standard templates.

Gene	Forward Primer Reverse Primer	Sequences	Annealing temperature (°C)	Amplification product (bp)
ABL	fABL_StdF	GGCTTTGAGGGAGACAAGAC	62	402
	fABL_StdR	GAAGCTGCCATTGATCAGAC		
ACTB	fACTB_StdF	CCATCGAACACGGCATTGTCAC	58	431
	fACTB_StdR	CTTGATGTCACGCACAATTTCCCG		
B2M	fB2M-F	GGCGCGTTTTGTGGTCTTGGTC	63	339
	fB2M-R	CACTTAACGACCTTGGGCTC		
GUSB	fGUSB_StdF	GCCGCATTACCATTGCCATCAAC	64	384
	fGUSB_StdR	GCATCAGGTATGGCCACCAGAG		
HMBS	fHMBS_StdF	CAGCCCAAAGATGAGAGTGATTCG	64	339
	fHMBS_StdR	GGGTGAAAGACAACGGCATCATAG		
RPS7	fRPS7-F	AGCTGAGGGAGCTGAACATC	65	432
	fRPS7-R	TGCCCGTGAGCTTCTTATAG		
YWHAZ	fYWHAZ_StdF	GAGGTTGCTGCTGGTGATGAC	64	329
	fYWHAZ_StdR	CCTGCTTCAGCTTCATCTCCTTGG		

TABLE 4. Optimal final concentration of primers and probe for the newly designed real-time PCR assays

Real-time PCR assay	Forward Primer (nM)	Reverse Primer (nM)	Probe (nM)
ABL	300	900	50
ACTB	900	900	50
B2M	300	900	100
GUSB	900	300	100
HMBS	900	900	250
HPRT	900	900	50
RPS7	50	900	200
YWHAZ	900	900	150

TABLE 5. Ranking of potential reference genes according to the expression stability. The expression stability is given as stability values calculated by NormFinder (NF), M values calculated by geNorm (gN(M)), and geNorm calculations (gN). Rankings are shown from most (left) to least (right) stable genes.

Tissues	Analsysis	Ranking
All endocrine tissues tested	NF	YWHAZ > GUSB > B2M > RPS7> HMBS > ABL > 18S > GAPDH > ACTB > HPRT
	gN(M)	B2M > GUSB > YWHAZ > HMBS > RPS7 > ABL > 18S > GAPDH > ACTB > HPRT
	gN	B2M = HMBS > GUSB > YWHAZ > RPS7 > ABL > 18S > GAPDH > ACTB > HPRT
Adrenal gland	NF	HPRT > RPS7 > ABL > B2M > ACTB > GAPDH > 18S > HMBS > YWHAZ > GUSB
	gN(M)	HPRT > RPS7 > ABL > ACTB > B2M > 18S = GAPDH > HMBS > YWHAZ > GUSB
	gN	GAPDH = 18S > RPS7 > HPRT > YWHAZ > ABL > B2M > ACTB > HMBS > GUSB
Pancreas	NF	RPS7 > GUSB > YWHAZ > HMBS > B2M > GAPDH > ABL > 18S > HPRT > ACTB
	gN(M)	RPS7 > GUSB > YWHAZ > B2M > HMBS > ABL > GAPDH > 18S > HPRT > ACTB
	gN	GUSB = RPS7 > YWHAZ > HMBS > B2M > ABL > 18S > GAPDH > HPRT > ACTB
Parathyroid	NF	ACTB > RPS7 > GAPDH > HMBS > YWHAZ > ABL > B2M > GUSB > 18S > HPRT
	gN(M)	ACTB > RPS7 > GAPDH > ABL > HMBS > YWHAZ > B2M > GUSB > 18S > HPRT
	gN	GAPDH = RPS7 > ACTB > HMBS > ABL > YWHAZ > B2M > GUSB > 18S > HPRT
Thyroid	NF	GUSB > ACTB > YWHAZ > HMBS > B2M > RPS7 > ABL > 18S > GAPDH > HPRT
	gN(M)	ACTB > GUSB > YWHAZ > B2M > HMBS > RPS7 > ABL > 18S > GAPDH > HPRT
	gN	ACTB = HMBS > GUSB > B2M > YWHAZ > RPS7 > ABL > 18S > GAPDH > HPRT
All lymphoid tissues tested	NF	RPS7 > GUSB > ACTB > YWHAZ > ABL > GAPDH > B2M > HPRT > 18S > HMBS
	gN(M)	GUSB > RPS7 > ACTB > YWHAZ > ABL > B2M > GAPDH > HPRT > 18S > HMBS
	gN	GUSB = RPS7 > ACTB > YWHAZ > ABL > B2M > GAPDH > HPRT > 18S > HMBS
Bone marrow	NF	RPS7 > HMBS > ABL > GUSB > YWHAZ > ACTB > B2M > 18S > HPRT > GAPDH
	gN(M)	RPS7 > HMBS > ABL > GUSB > YWHAZ > ACTB > B2M > 18S > HPRT > GAPDH
	gN	ACTB = YWHAZ > HMBS > GUSB > RPS7 > ABL > B2M > 18S > HPRT > GAPDH
Lymph node	NF	GUSB > ACTB > B2M > HMBS > YWHAZ > ABL > RPS7 > GAPDH > 18S > HPRT
	gN(M)	GUSB > B2M > ACTB > YWHAZ > ABL > HMBS > RPS7 > GAPDH > 18S > HPRT
	gN	ABL = B2M > YWHAZ > ACTB > GUSB > HMBS > GAPDH > RPS7 > 18S > HPRT
Spleen	NF	GUSB > RPS7 > ACTB > ABL > B2M > HMBS > YWHAZ > HPRT > GAPDH > 18S
	gN(M)	GUSB > RPS7 > ACTB > B2M > ABL > HMBS > YWHAZ > HPRT > GAPDH > 18S
	gN	ACTB = B2M > GUSB > RPS7 > ABL > HMBS > YWHAZ > HPRT > GAPDH > 18S
All gastrointest-inal tissues tested	NF	ACTB > HMBS > YWHAZ > RPS7 > ABL > GAPDH > GUSB > B2M > 18S > HPRT
	gN(M)	ACTB > HMBS > YWHAZ > RPS7 > ABL > GAPDH > GUSB > B2M > 18S > HPRT
	gN	ABL = HMBS > RPS7 > ACTB > YWHAZ > GAPDH > B2M > GUSB > 18S > HPRT

Parotid gland	NF	ABL > ACTB > RPS7 > YWHAZ > HMBS > B2M > HPRT > GAPDH > GUSB > 18S
	gN(M)	ABL > RPS7 > ACTB > YWHAZ > HMBS > B2M > HPRT > GAPDH > GUSB > 18S
	gN	ABL = ACTB > HMBS > RPS7 > YWHAZ > B2M > HPRT > GAPDH > GUSB > 18S
Duodenum	NF	RPS7 > GAPDH > ACTB > HMBS > YWHAZ > ABL > 18S > GUSB > B2M > HPRT
	gN(M)	RPS7 > GAPDH > ACTB > HMBS > YWHAZ > ABL > 18S > GUSB > B2M > HPRT
	gN	ACTB = HMBS > RPS7 > GAPDH > YWHAZ > ABL > 18S > GUSB > B2M > HPRT
Ileum	NF	HMBS > ABL > YWHAZ > ACTB > GAPDH > GUSB > RPS7 > HPRT > B2M > 18S
	gN(M)	HMBS > ABL > ACTB > YWHAZ > GAPDH > GUSB > RPS7 > HPRT > B2M > 18S
	gN	YWHAZ = ACTB > HMBS > ABL > GUSB > GAPDH > RPS7 > HPRT > B2M > 18S
Liver	NF	GUSB > GAPDH > RPS7 > HPRT > HMBS > YWHAZ > ABL > ACTB > B2M > 18S
	gN(M)	GAPDH > HMBS > HPRT > GUSB > RPS7 > YWHAZ > ABL > ACTB > B2M > 18S
	gN	ACTB = HMBS > ABL > GAPDH > HPRT > GUSB > YWHAZ > RPS7 > B2M > 18S
Kidney	NF	YWHAZ > RPS7 > ABL > ACTB > HMBS > GUSB > GAPDH > B2M > HPRT > 18S
	gN(M)	YWHAZ > RPS7 > ABL > ACTB > HMBS > GUSB > GAPDH > B2M > HPRT > 18S
	gN	RPS7 = YWHAZ > ABL > ACTB > HMBS > GAPDH > GUSB > B2M > HPRT > 18S
Myocardium	NF	ACTB > RPS7 > GAPDH > HMBS > YWHAZ > ABL > GUSB > 18S > HPRT > B2M
	gN(M)	RPS7 > ACTB > GAPDH > YWHAZ > HMBS > ABL > GUSB > 18S > HPRT > B2M
	gN	GAPDH = YWHAZ > RPS7 > ACTB > HMBS > ABL > GUSB > 18S > HPRT > B2M
Brain	NF	ACTB > ABL > RPS7 = YWHAZ > HMBS > GAPDH > HPRT > GUSB > B2M > 18S
	gN(M)	ACTB > ABL > RPS7 > YWHAZ > HPRT > GAPDH > HMBS > GUSB > B2M > 18S
	gN	ABL = RPS7 > ACTB > GAPDH > HPRT > YWHAZ > HMBS > GUSB > B2M > 18S
All healthy tissues combined	NF	RPS7 > GUSB > YWHAZ > ABL > ACTB > B2M > HMBS > 18S > GAPDH > HPRT
	gN(M)	RPS7 > GUSB > YWHAZ > ABL > ACTB > B2M > HMBS > 18S > GAPDH > HPRT
	gN	GUSB = RPS7 > ABL > YWHAZ > ACTB > B2M > HMBS > 18S > GAPDH > HPRT
Blood	NF	YWHAZ > ABL > GAPDH > B2M > ACTB > GUSB > 18S > HPRT > RPS7 > HMBS
	gN(M)	YWHAZ > B2M > GAPDH > ABL > ACTB > 18S > GUSB > HPRT > RPS7 > HMBS
	gN	YWHAZ = B2M > ACTB > 18S > GAPDH > ABL > GUSB > HPRT > RPS7 > HMBS
Neoplastic tissues	NF	RPS7 > ACTB > HMBS > GAPDH > B2M > ABL > 18S > GUSB > YWHAZ > HPRT
	gN(M)	ACTB > RPS7 > HMBS > GAPDH > B2M > 18S > ABL > GUSB > YWHAZ > HPRT
	gN	GAPDH = HMBS > 18S > RPS7 > ACTB > B2M > ABL > GUSB > YWHAZ > HPRT

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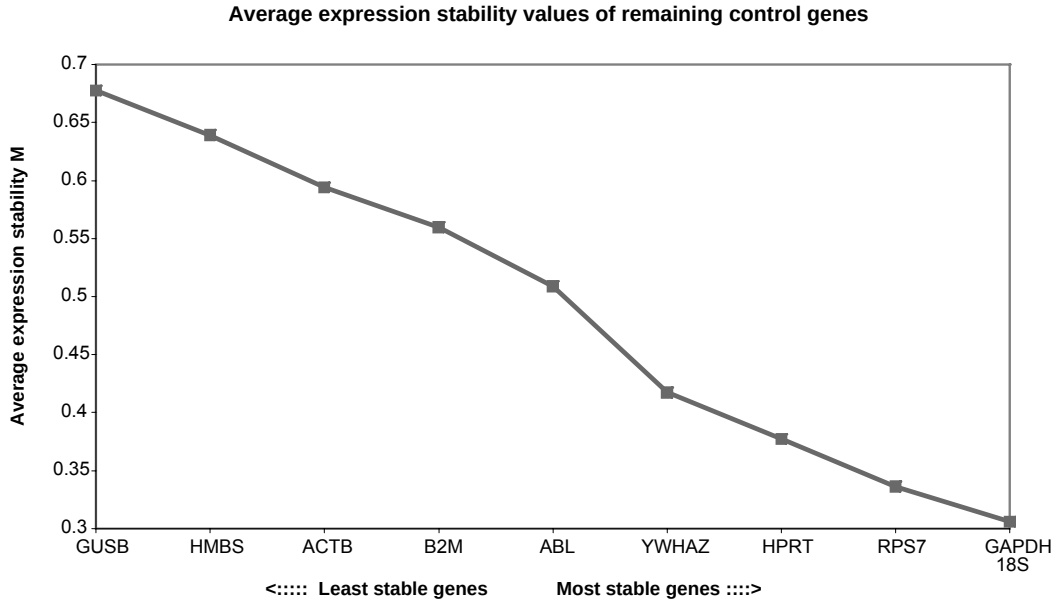
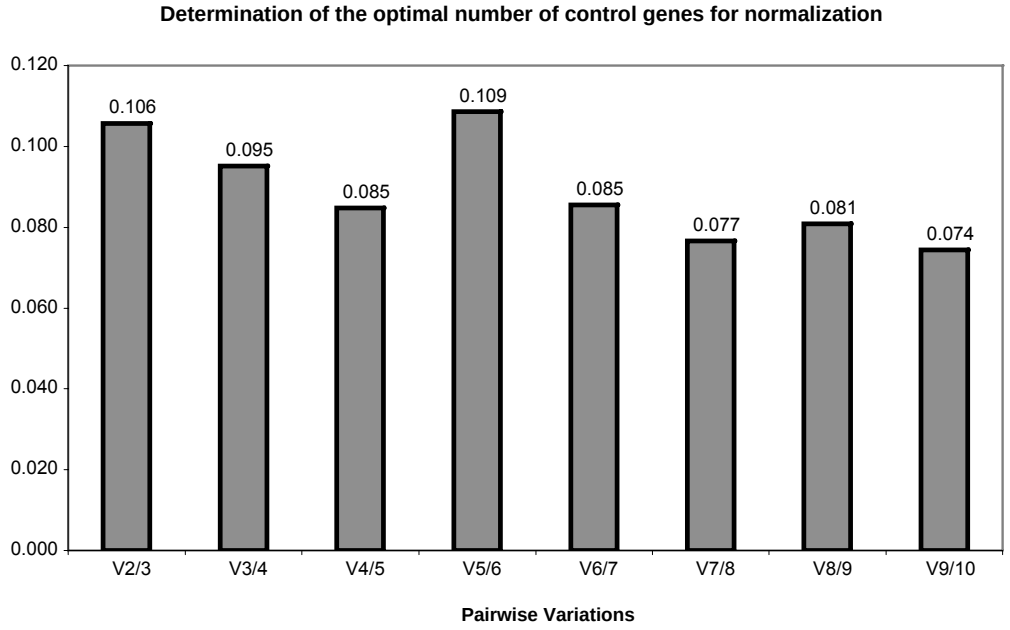
7 Appendix

Appendix 1: geNorm output files

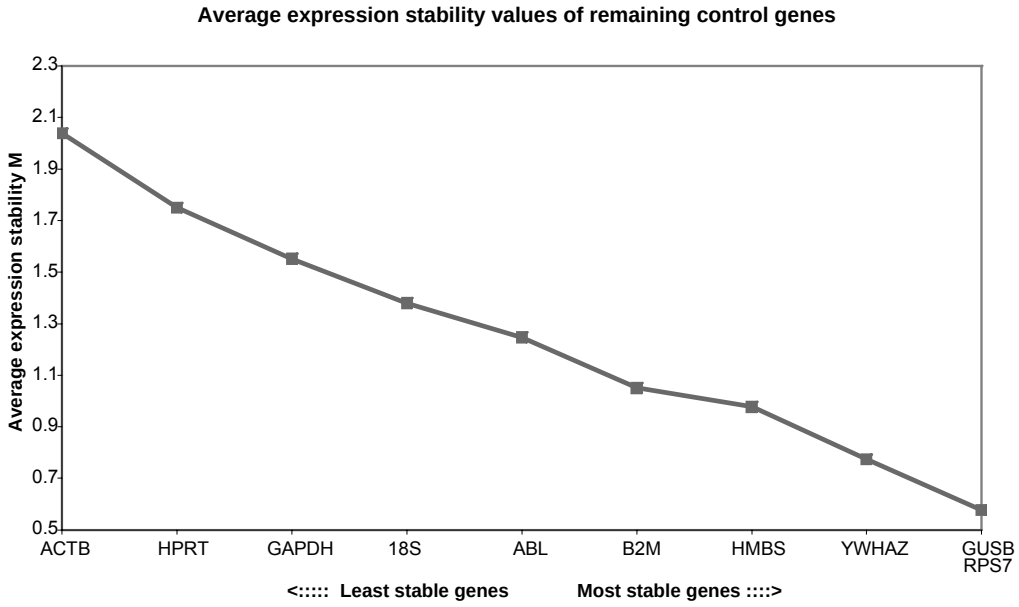
Average expression stability values calculated by the geNorm program for reference genes included in this study and graph showing the optimal number of reference genes for normalization. Output files are shown for a) adrenal gland, b) pancreas, c) parathyroid, d) thyroid, e) bone marrow, f) lymph node, g) spleen, h) parotid gland, i) duodenum, j) ileum, k) liver, l) kidney, m) myocardium, n) brain, o) blood, p) neoplastic tissues, q) all 14 healthy tissues combined, r) all endocrine tissues, s) all lymphoid tissues, and t) all gastrointestinal tissues.

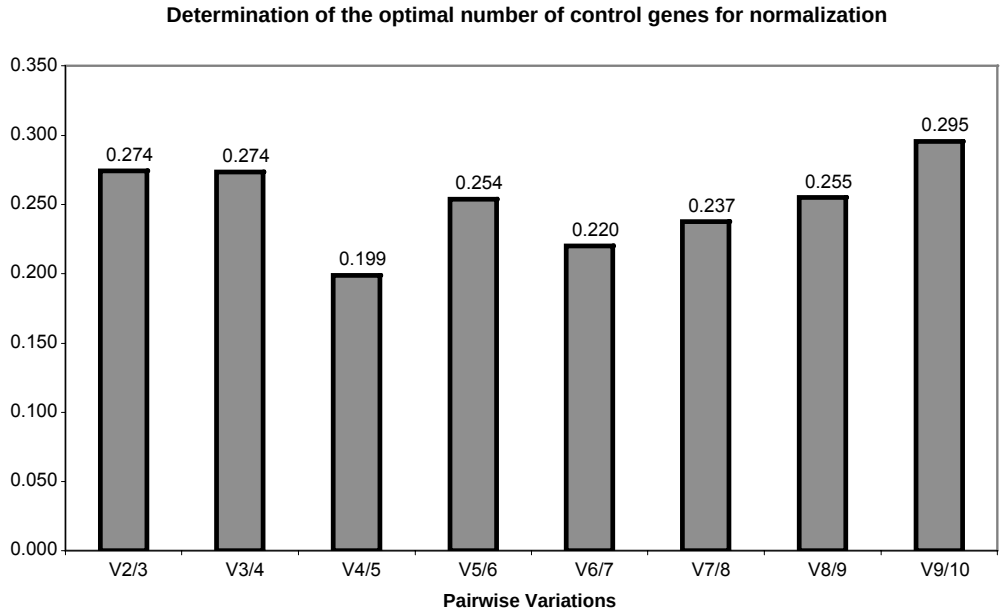
a) geNorm output: Adrenal gland



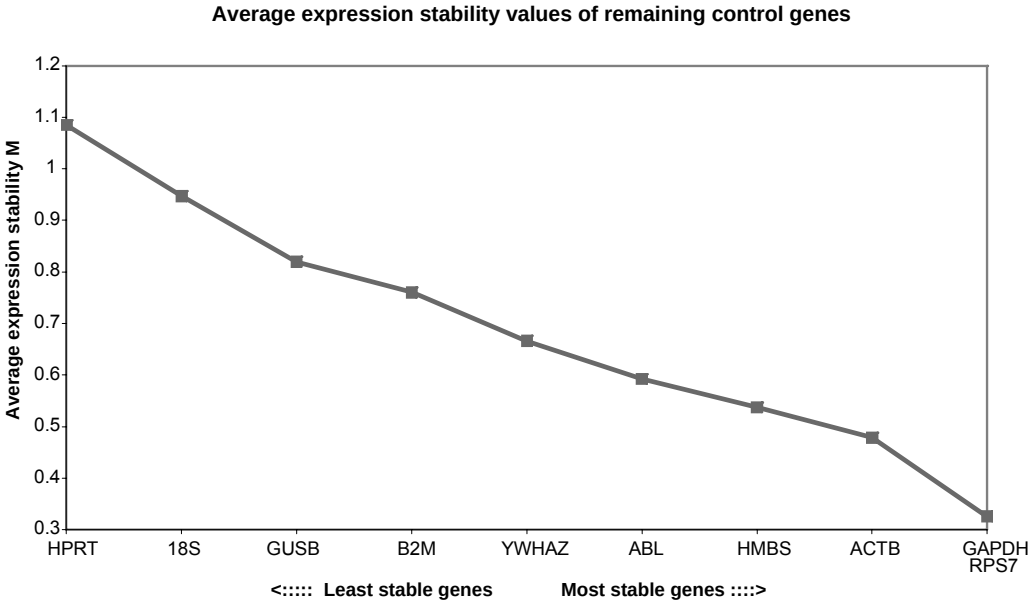


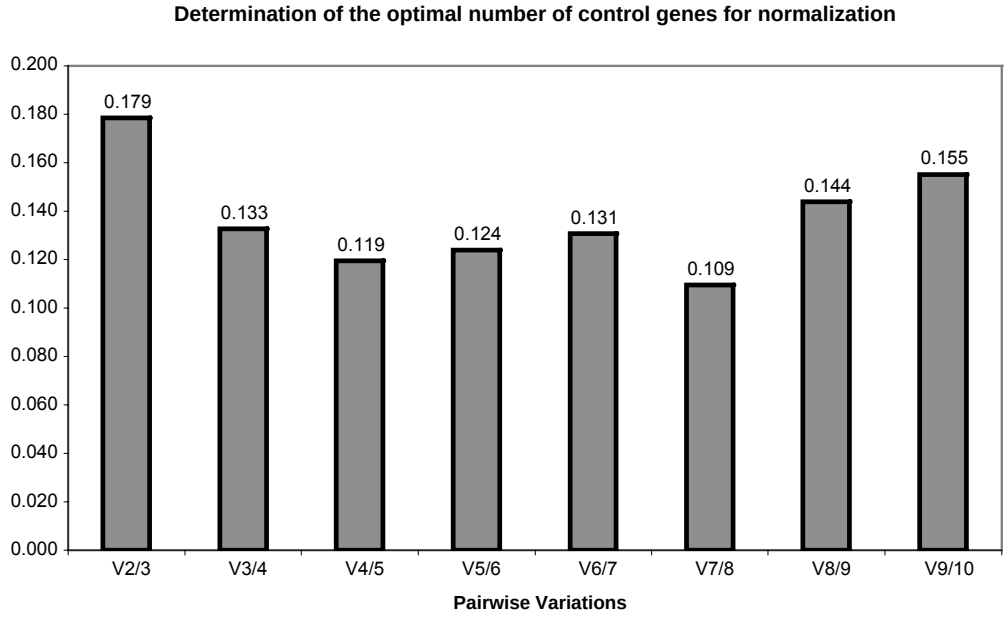
b) geNorm output: Pancreas



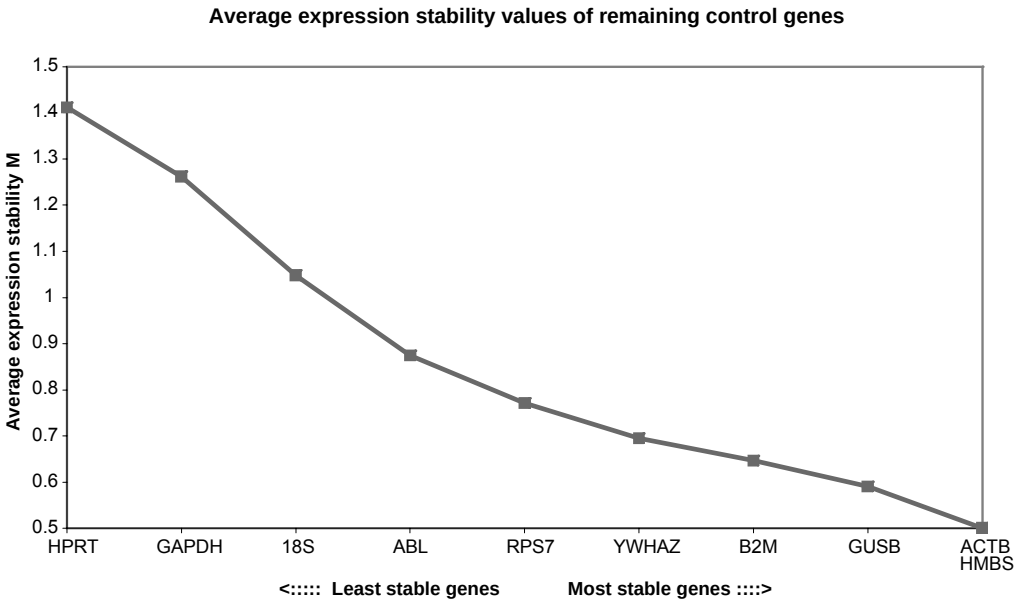


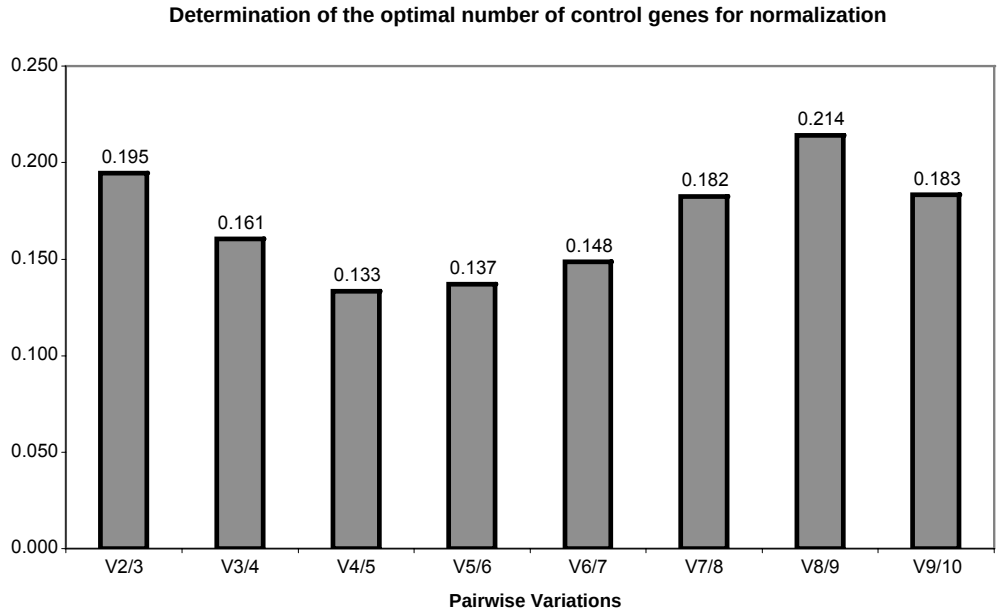
c) geNorm output: Parathyroid



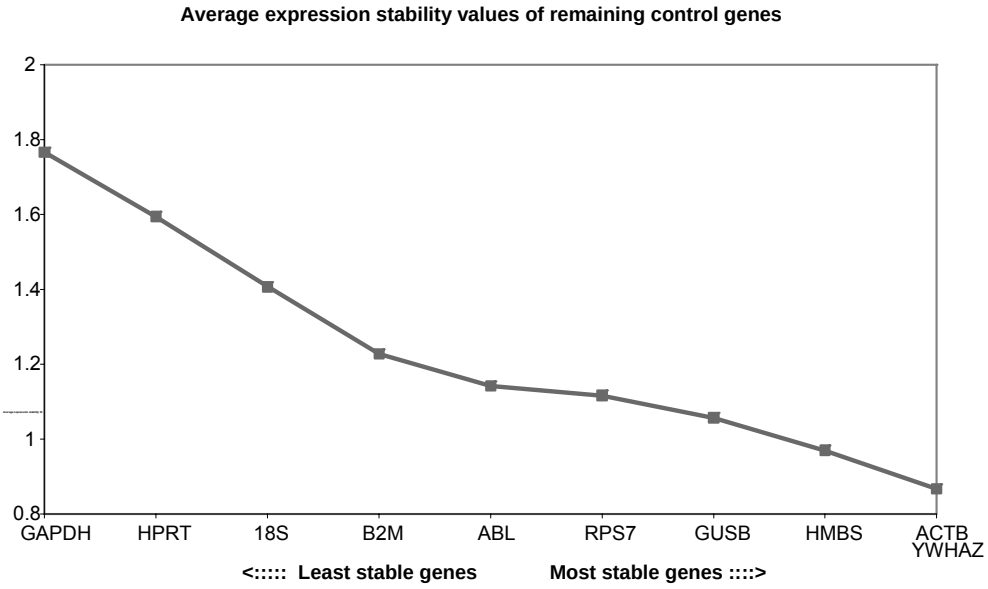


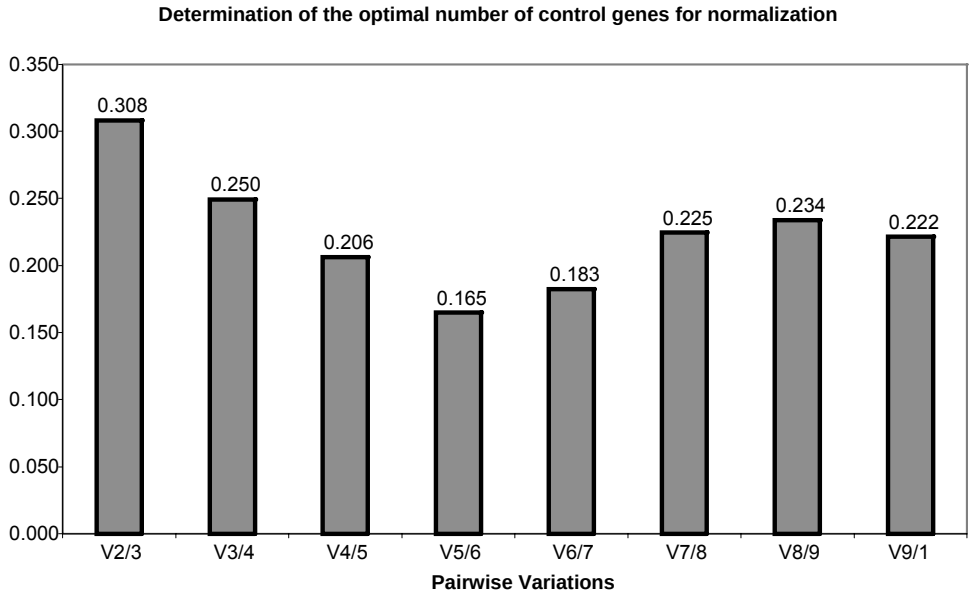
d) geNorm output: Thyroid



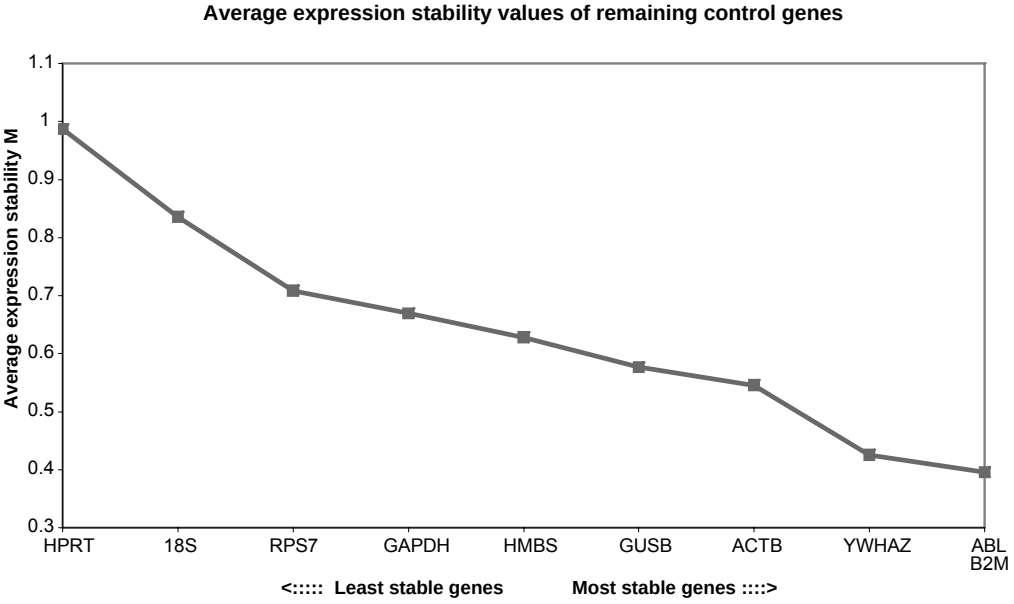


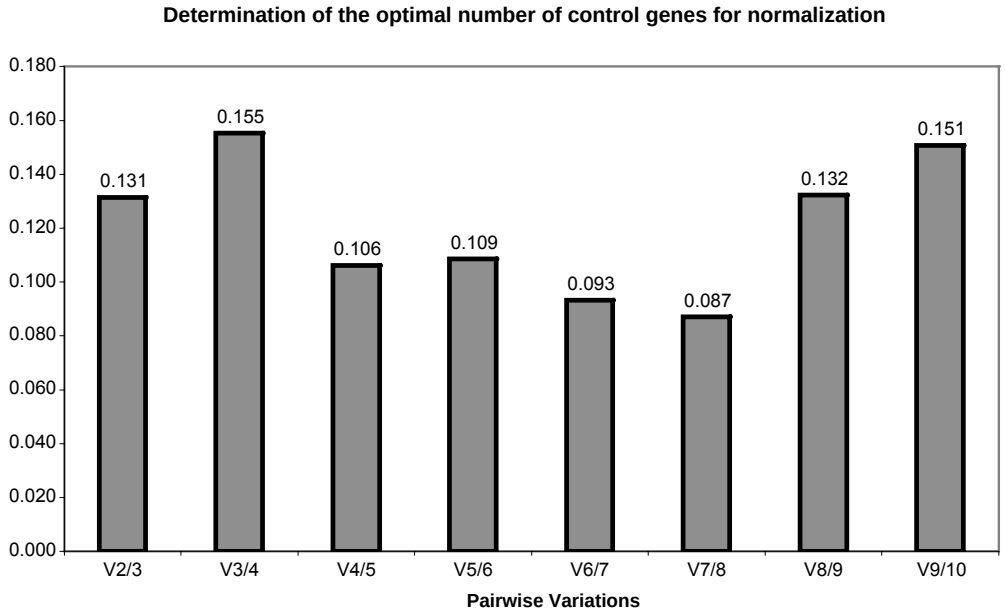
e) geNorm output: Bone marrow



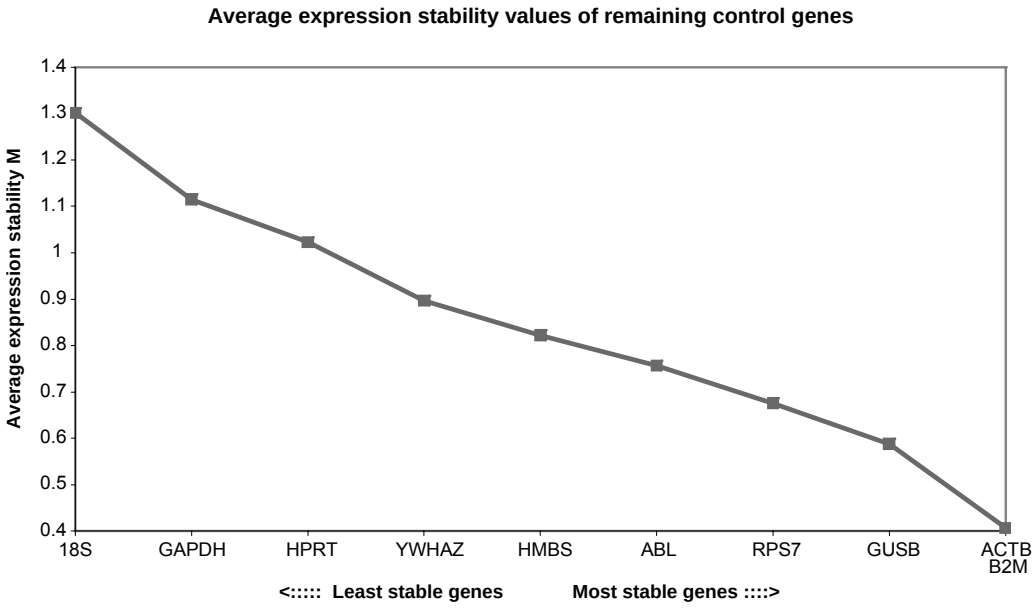


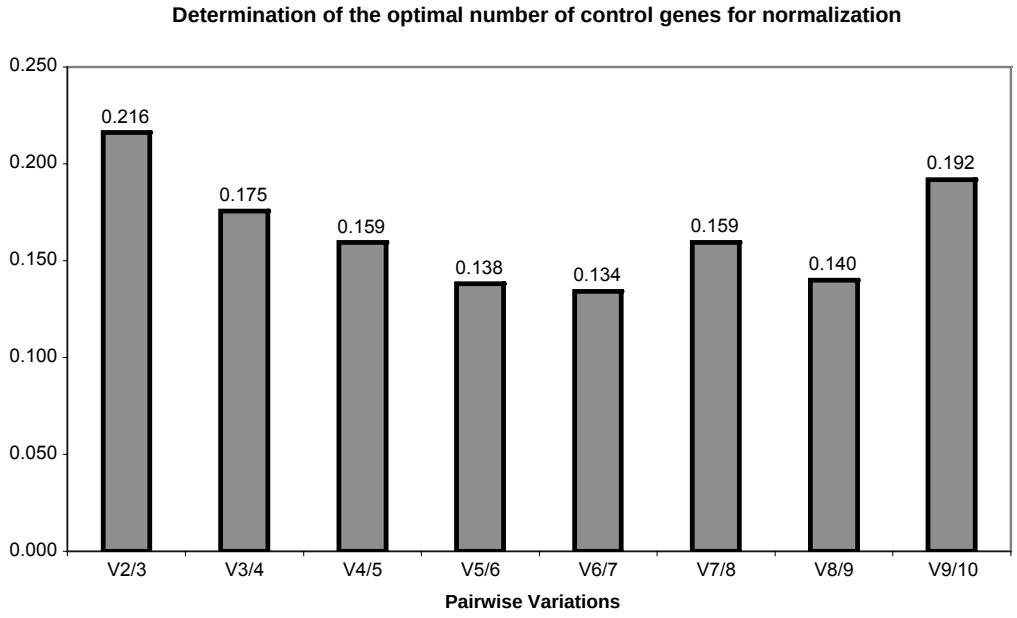
f) geNorm output: Mesenteric lymph node



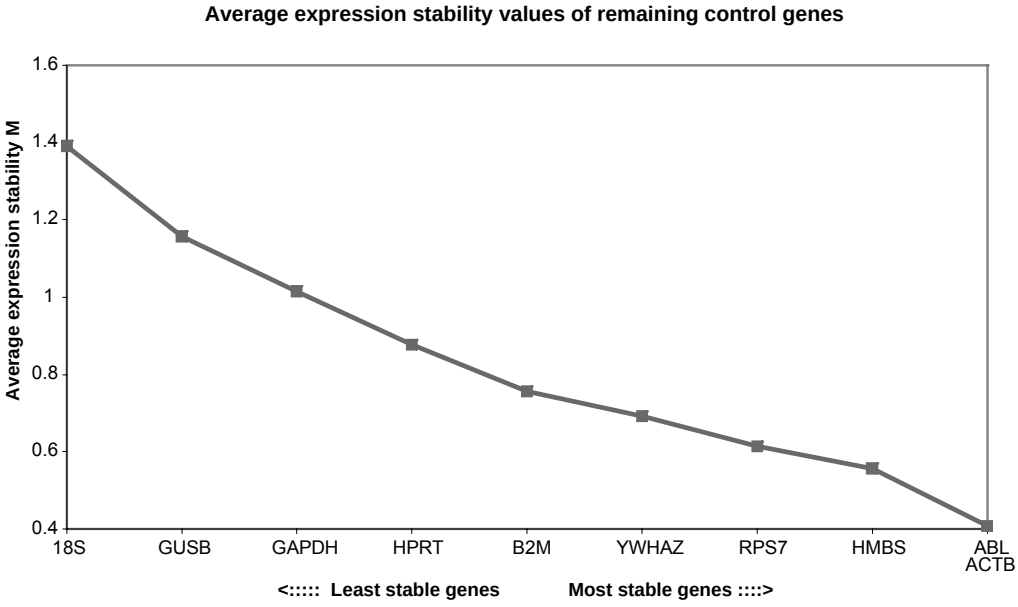


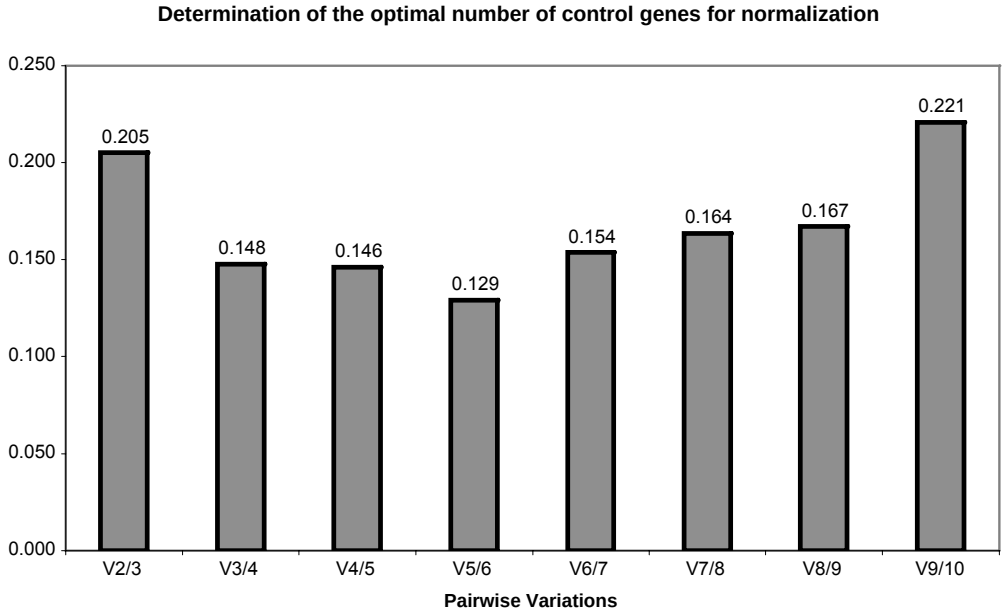
g) geNorm output: Spleen



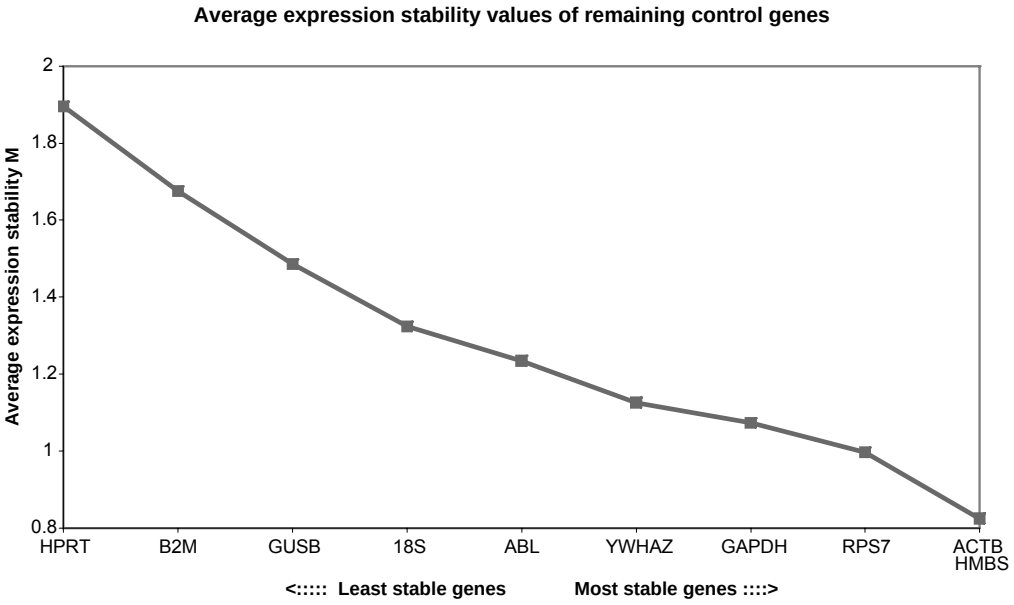


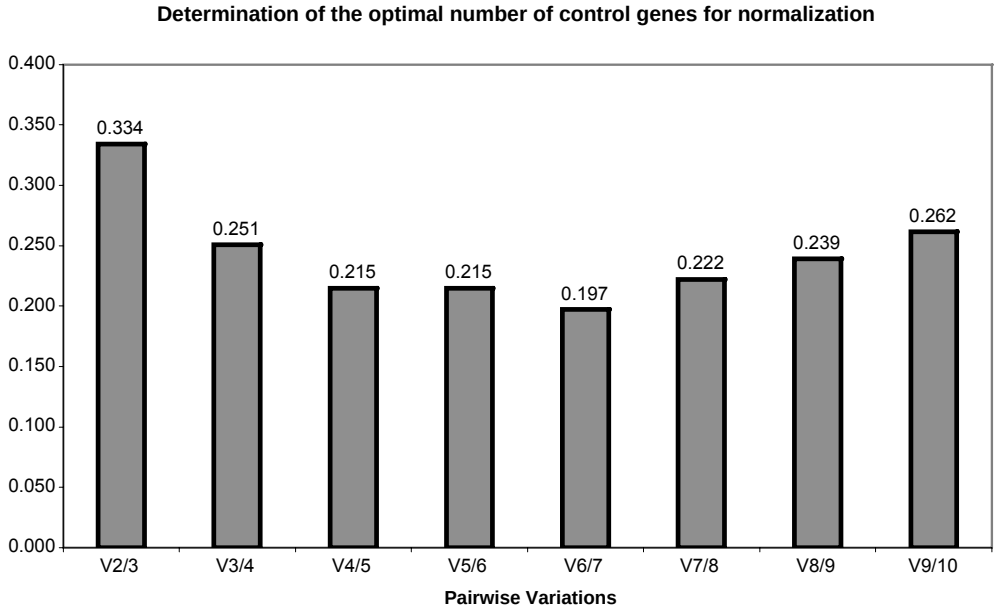
h) geNorm output: Parotid gland



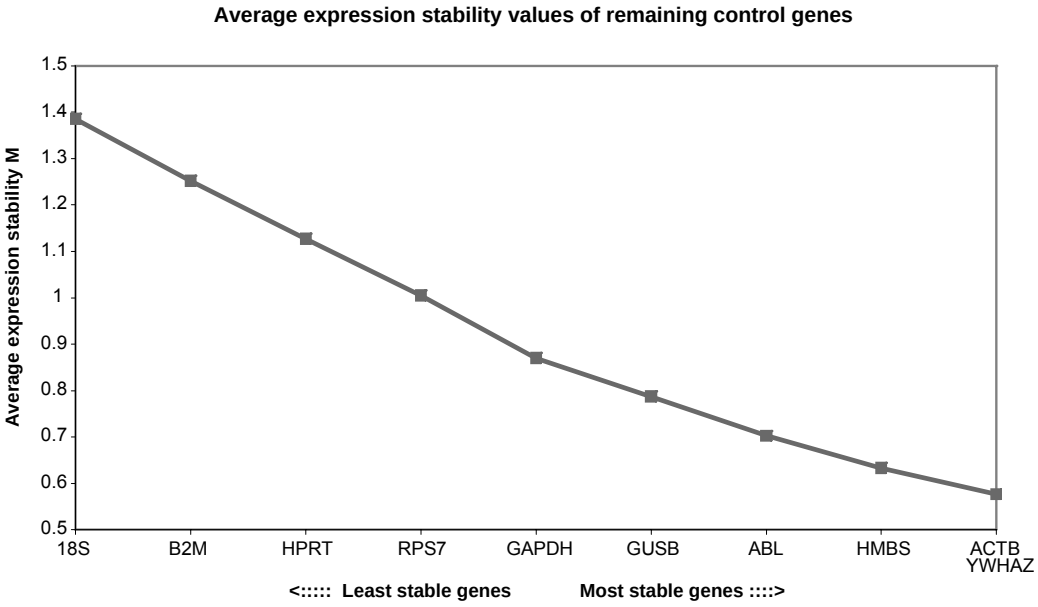


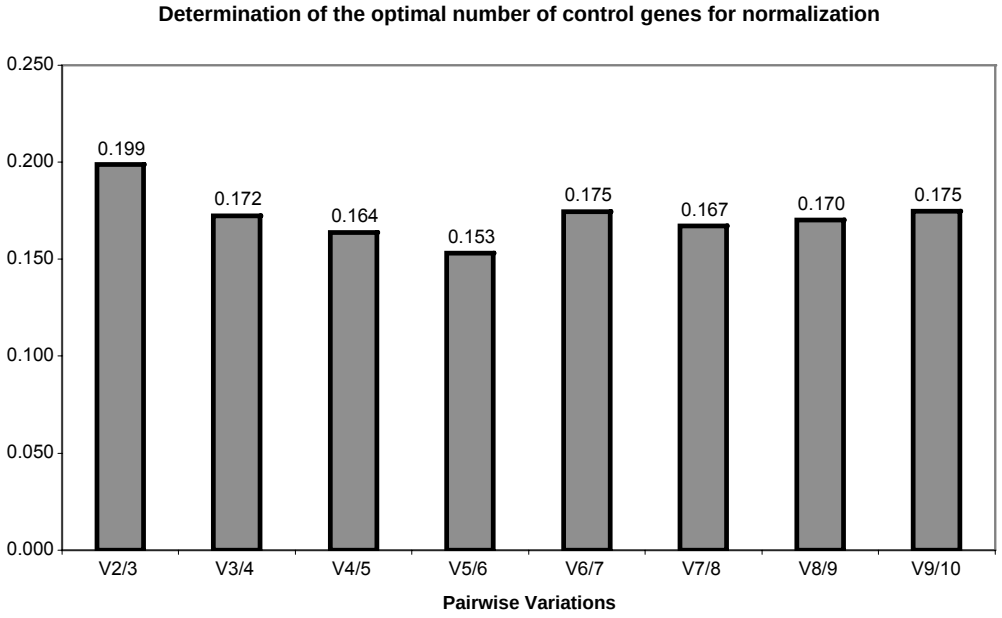
i) geNorm output: Duodenum



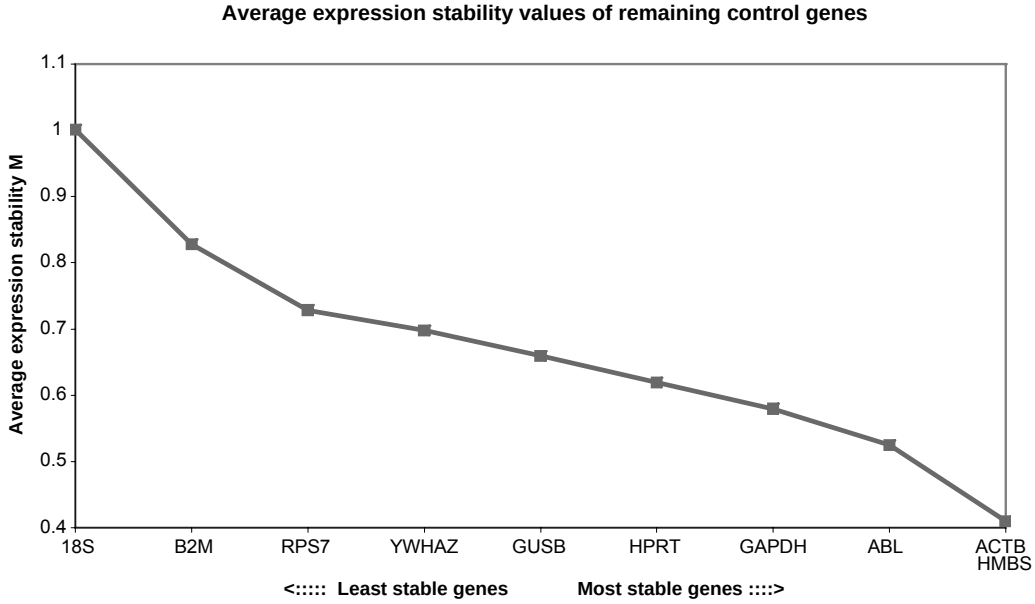


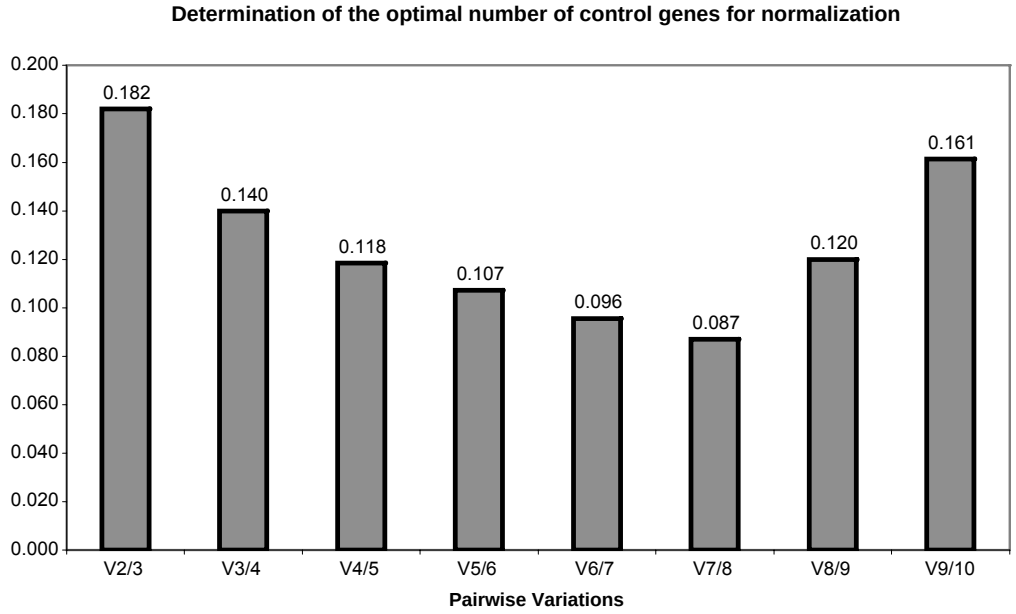
j) geNorm output: Ileum



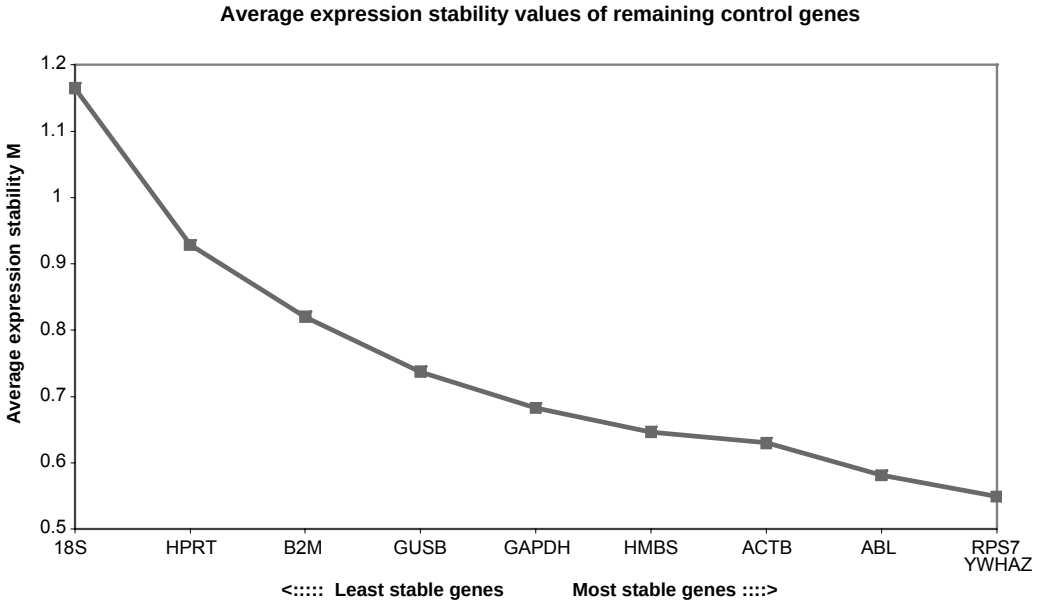


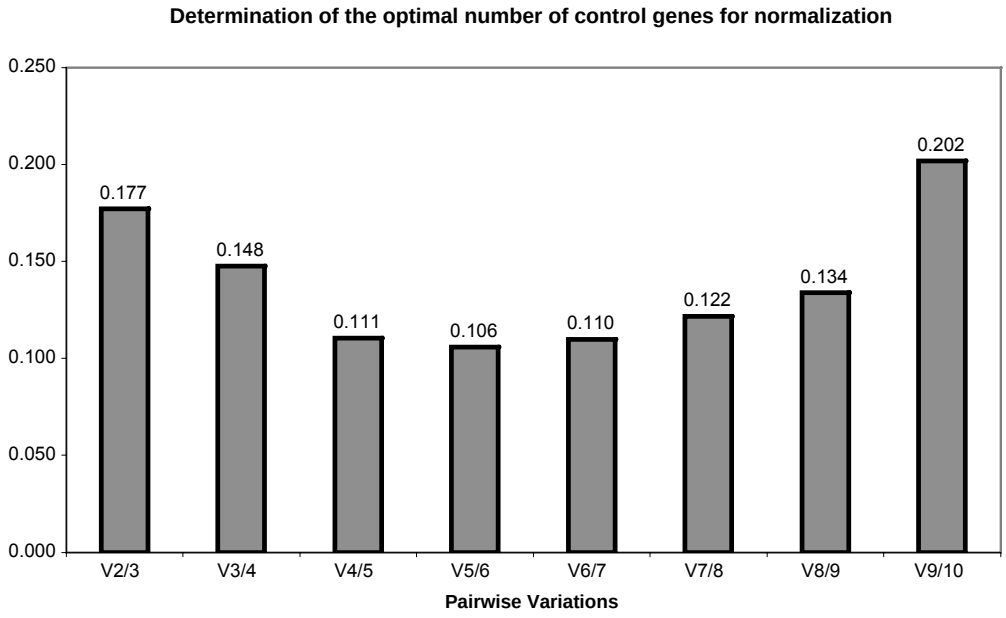
k) geNorm output: Liver



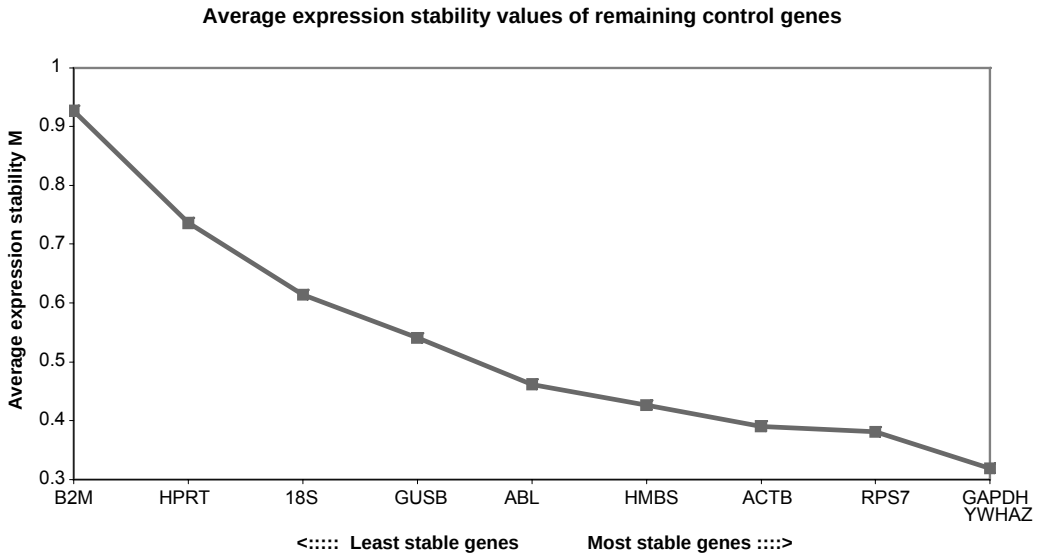


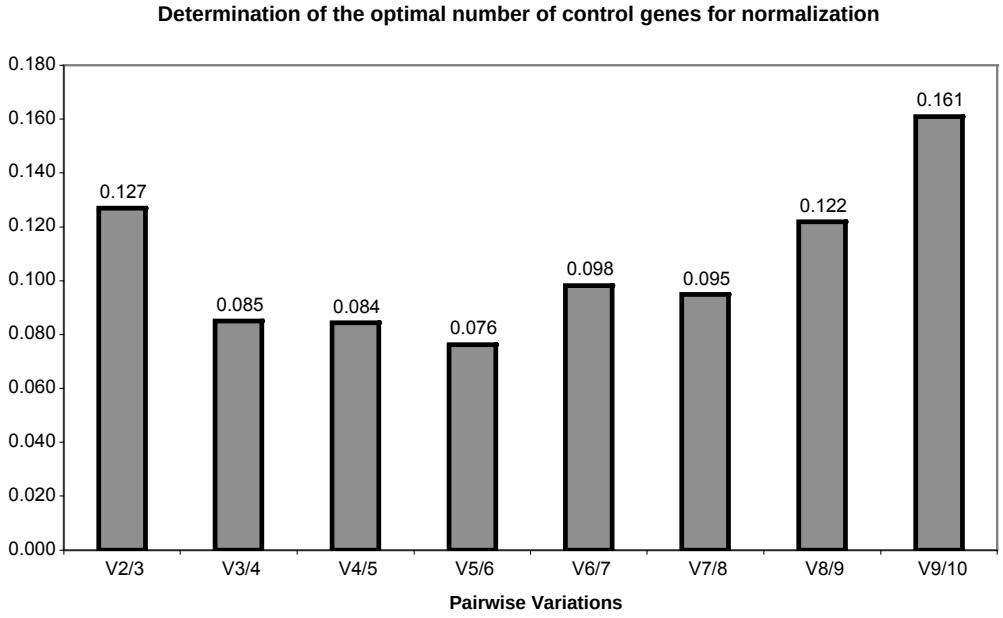
l) geNorm output: Kidney



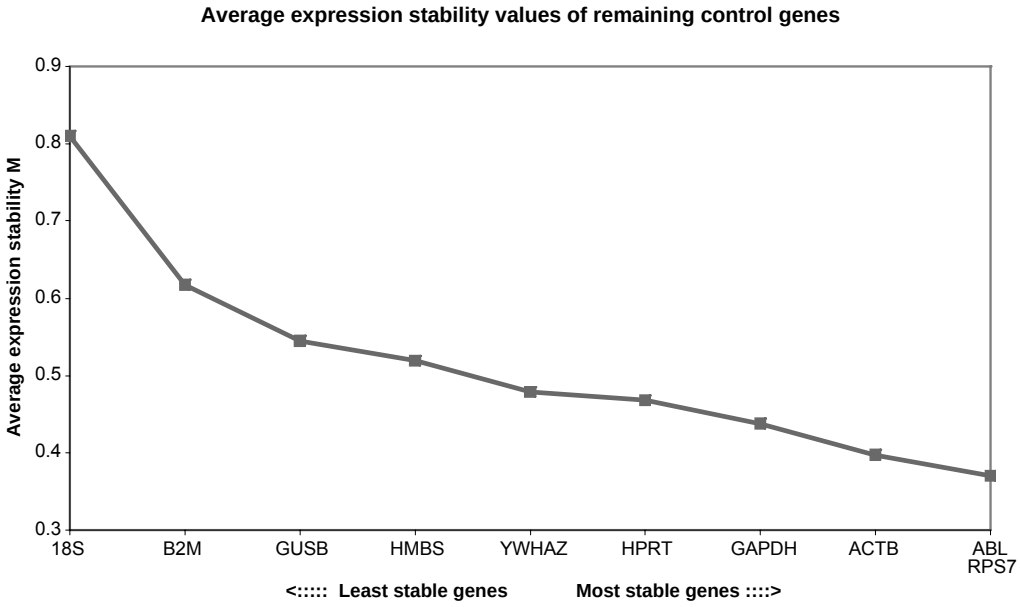


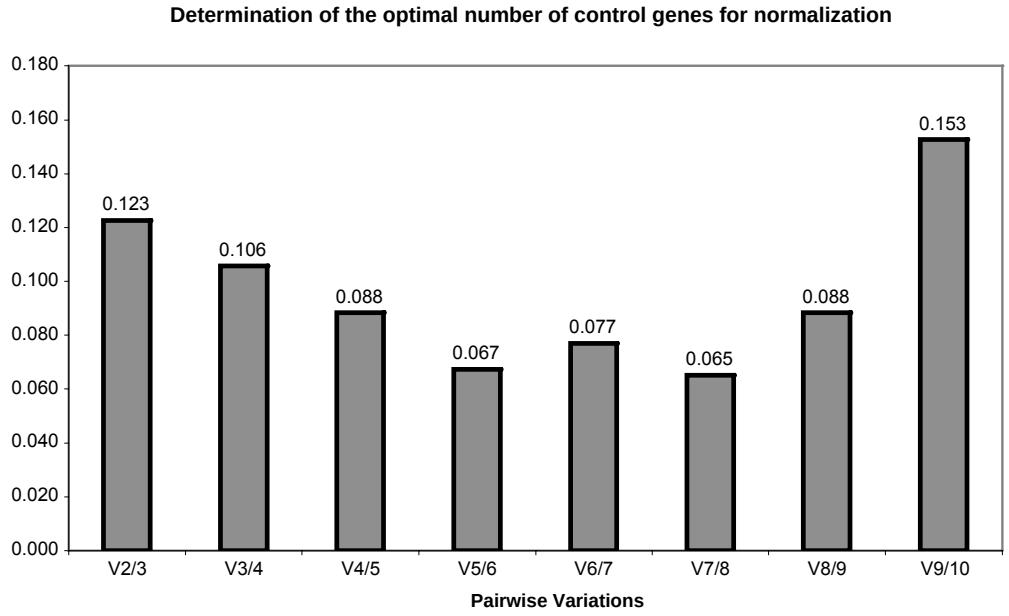
m) geNorm output: Myocardium



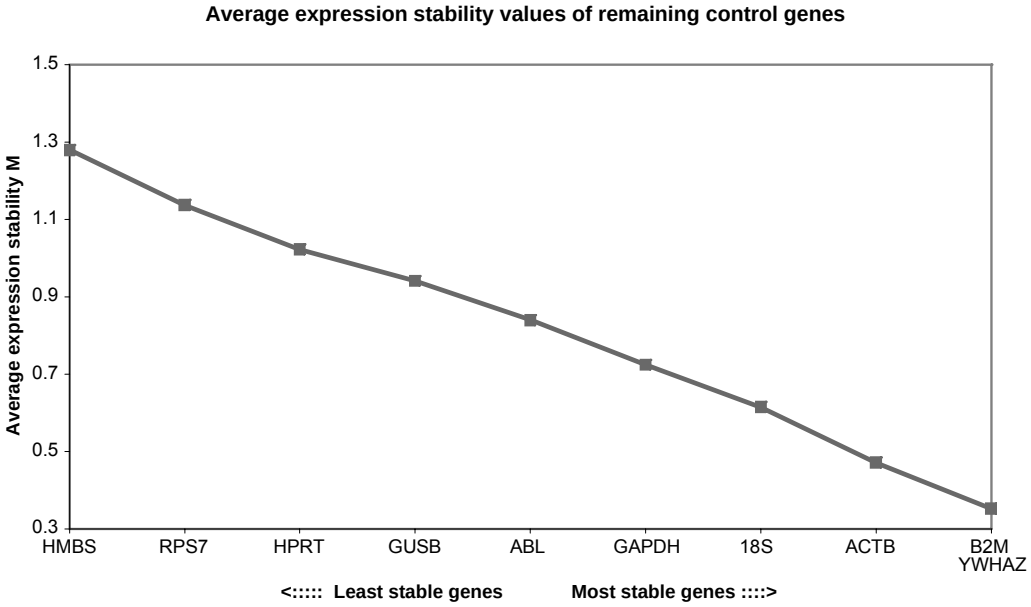


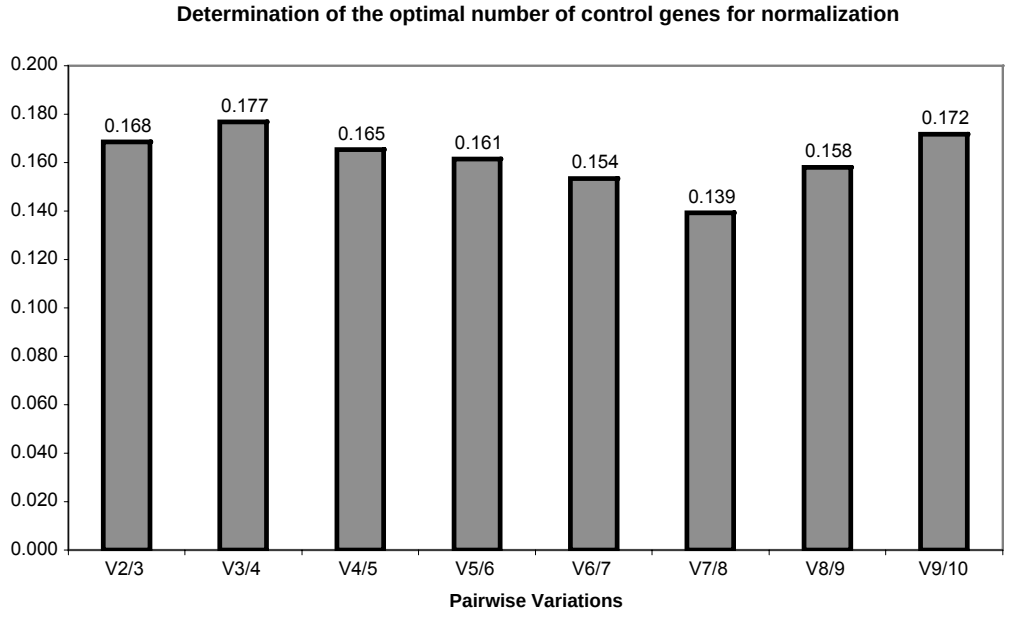
n) geNorm output: Brain



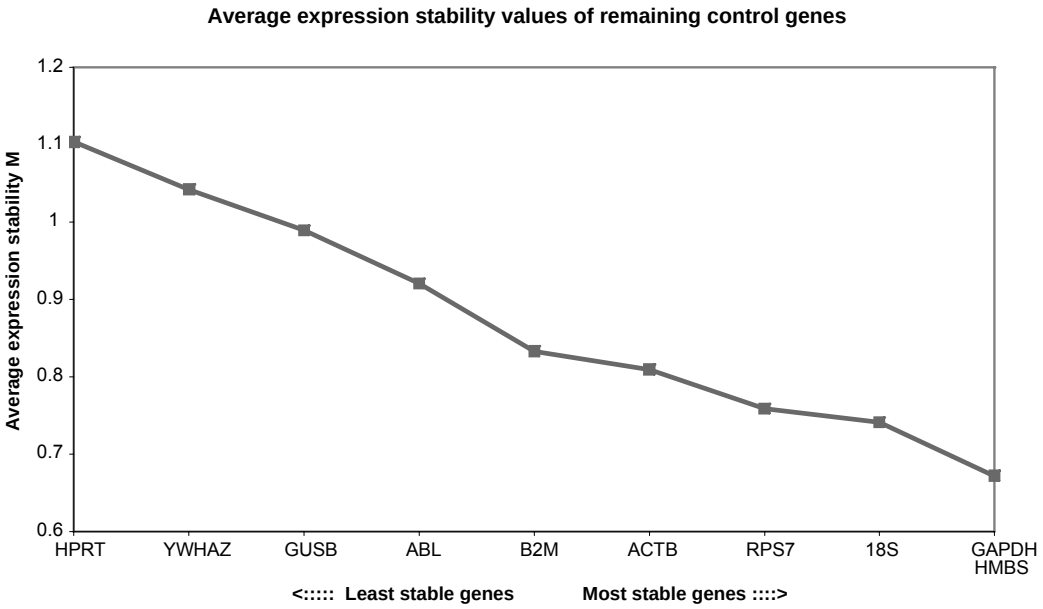


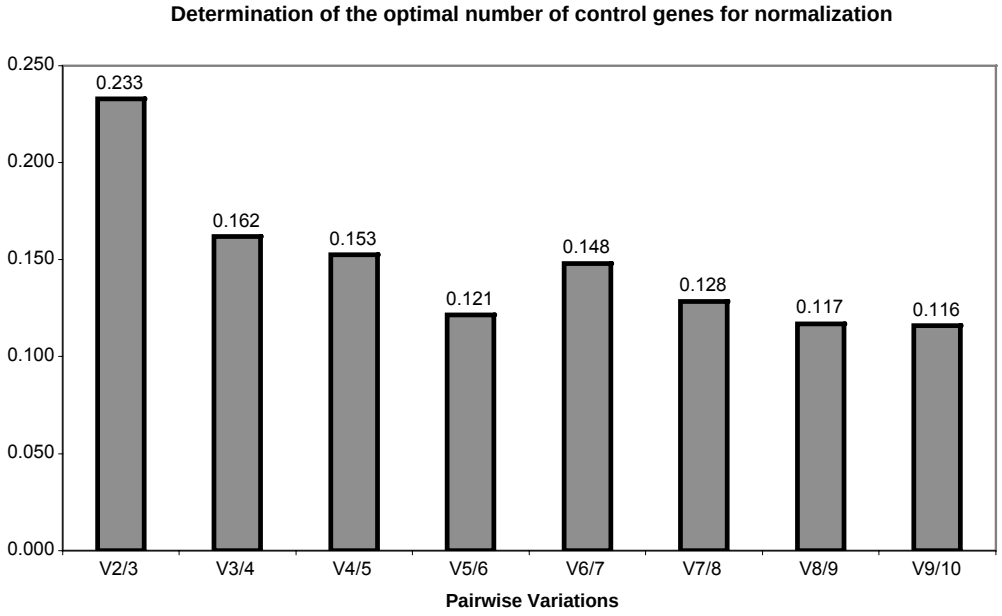
o) geNorm output: Blood



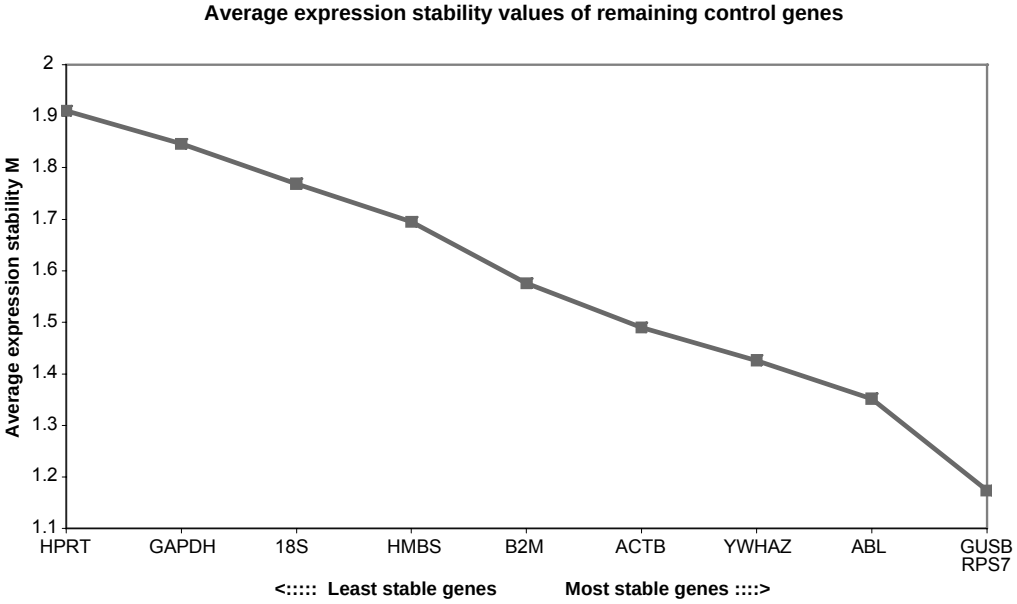


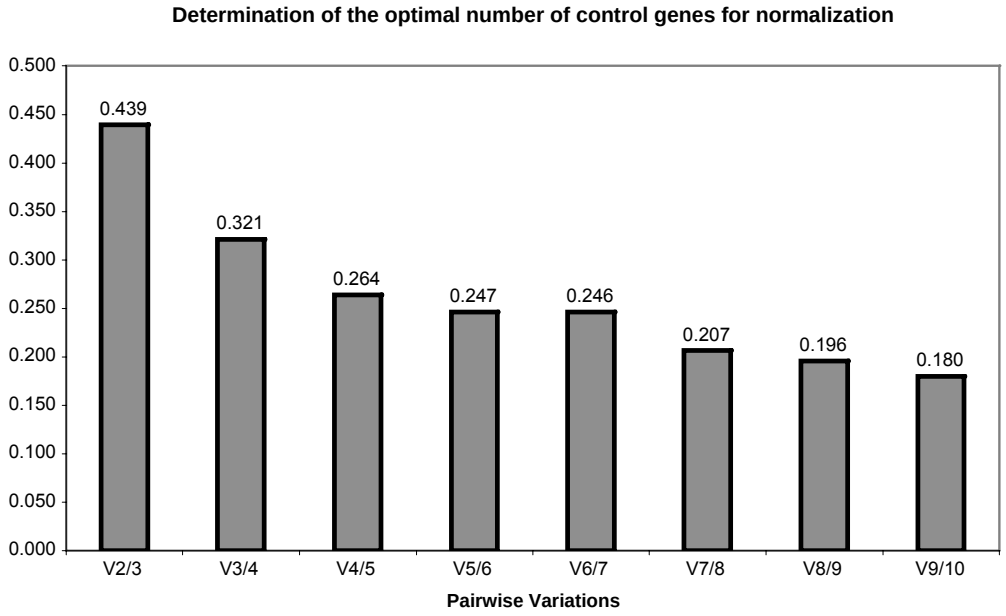
p) geNorm output: Neoplastic tissues



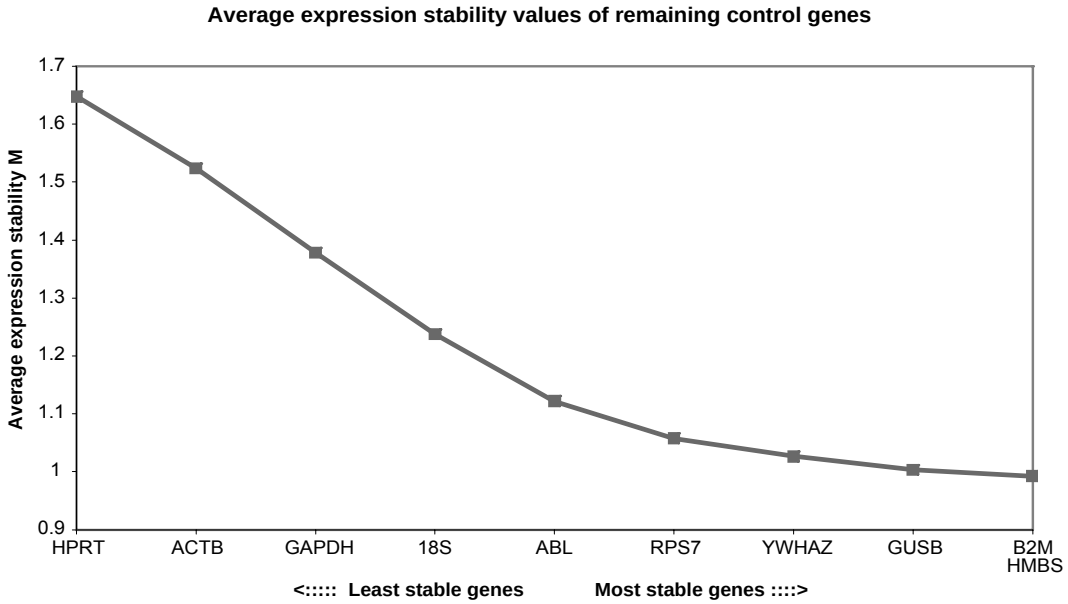


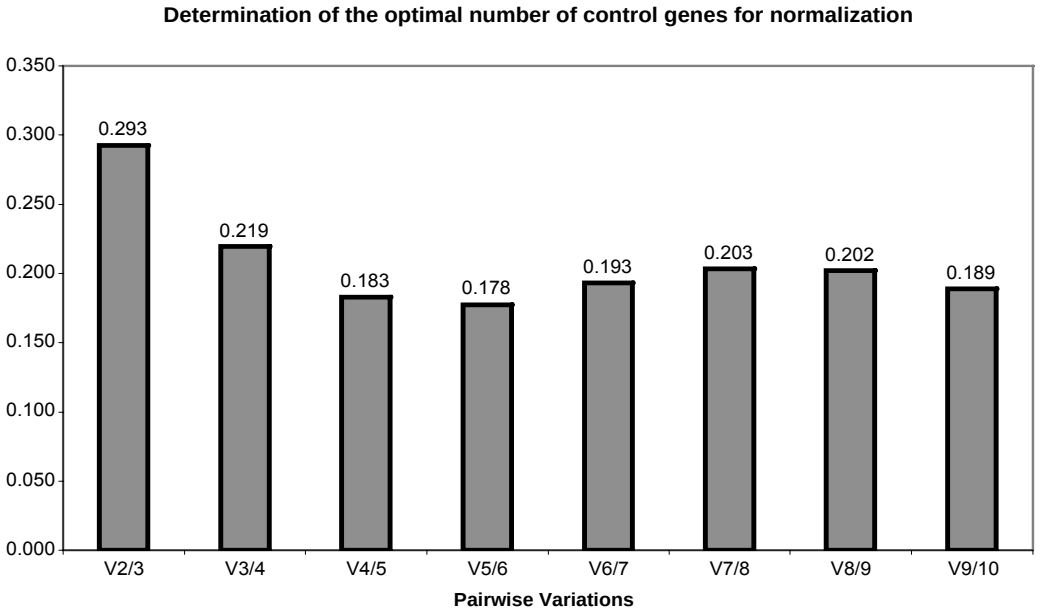
q) geNorm output: All healthy tissues



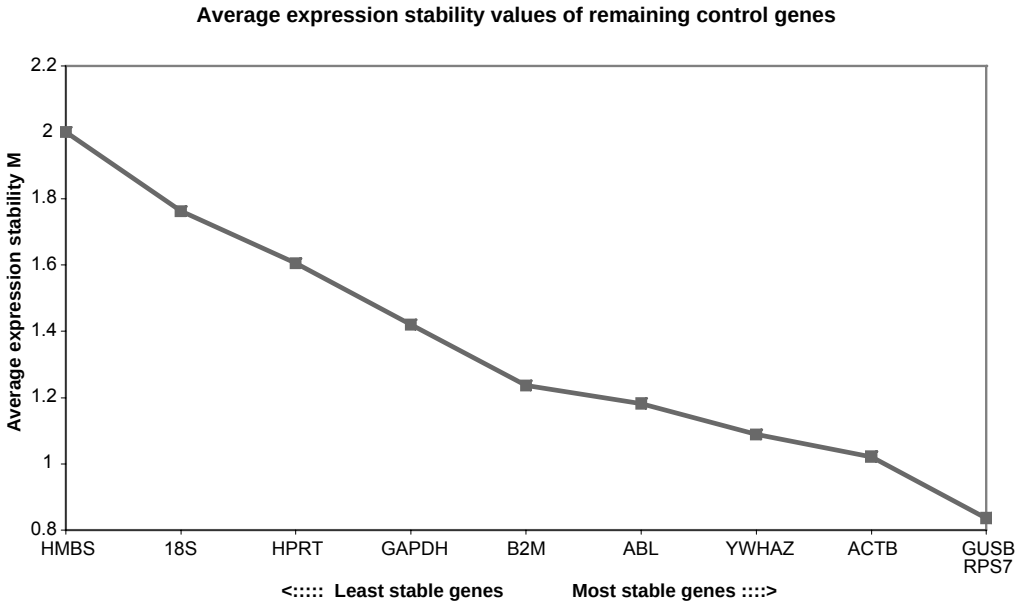


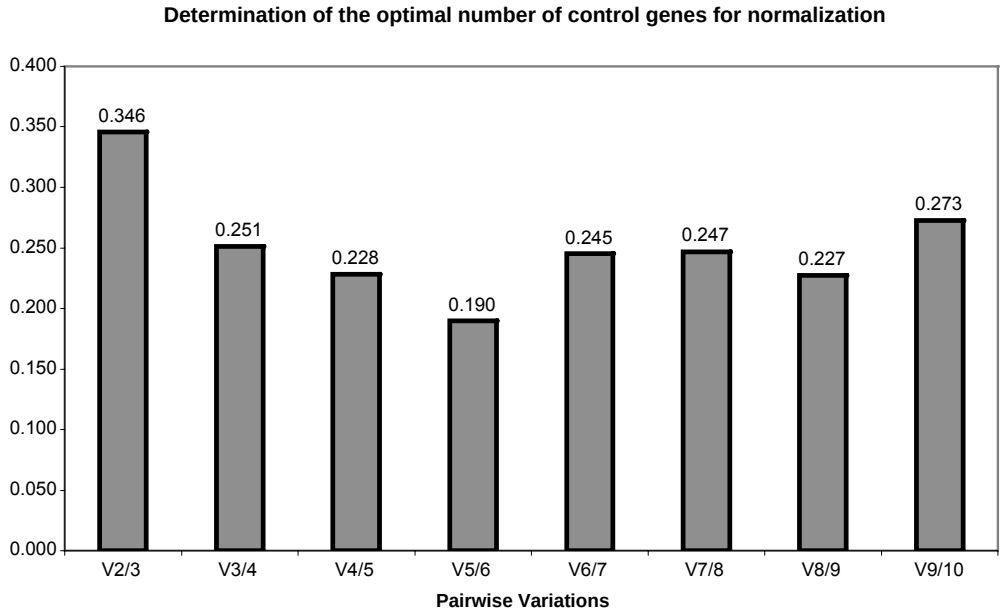
r) geNorm output: Endocrine tissues



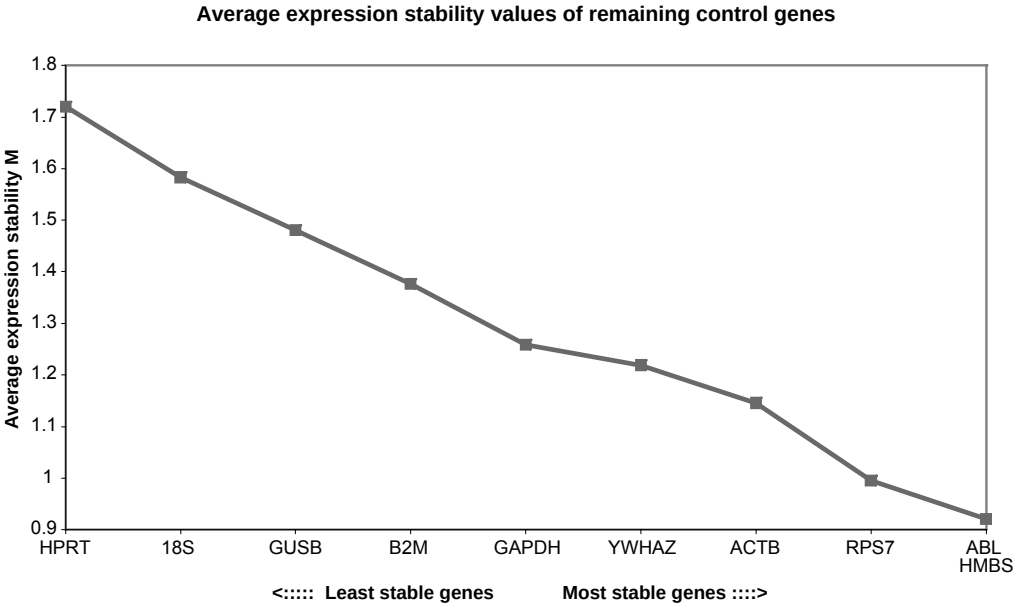


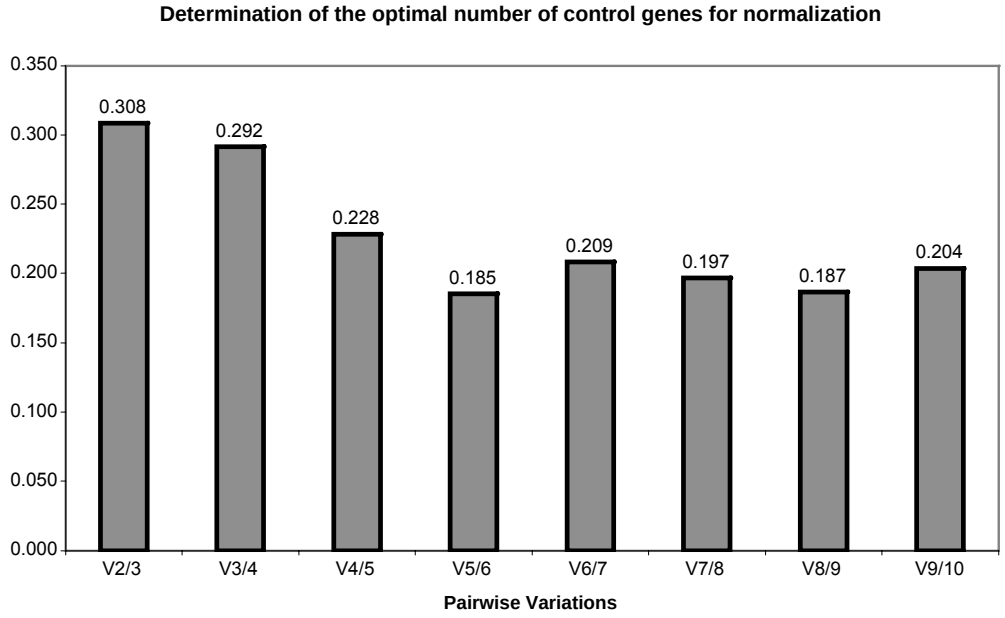
s) geNorm output: Lymphoid tissues





t) geNorm output: Gastrointestinal tissues





Appendix 2: NormFinder output

Stability values and standard errors calculated by the NormFinder program. In addition, a gene ranking for every tissue is shown

Lymphatic tissues				**Endocrine tissues**			
Gene name	Stability value	Standard error	Ranking	Gene name	Stability value	Standard error	Ranking
ABL	0.991	0.144	5	ABL	0.802	0.101	6
ACTB	0.636	0.108	3	ACTB	1.227	0.143	9
GAPDH	1.036	0.149	6	GAPDH	1.028	0.123	8
B2M	1.078	0.153	7	B2M	0.464	0.072	3
GUSB	0.301	0.096	2	GUSB	0.462	0.072	2
HMBS	1.867	0.247	10	HMBS	0.518	0.076	5
HPRT	1.204	0.168	8	HPRT	1.284	0.149	10
RPS7	0.278	0.098	1	RPS7	0.509	0.075	4
YWHAZ	0.707	0.115	4	YWHAZ	0.450	0.072	1
18S	1.273	0.176	9	18S	1.023	0.123	7
Gastrointestinal tissues				**All tissues**			
Gene name	Stability value	Standard error	Ranking	Gene name	Stability value	Standard error	Ranking
ABL	0.626	0.103	5	ABL	0.816	0.055	4
ACTB	0.490	0.091	1	ACTB	0.881	0.058	5
GAPDH	0.700	0.111	6	GAPDH	1.186	0.074	9
B2M	1.099	0.157	8	B2M	1.019	0.065	6
GUSB	1.008	0.146	7	GUSB	0.693	0.050	2
HMBS	0.546	0.095	2	HMBS	1.025	0.066	7
HPRT	1.387	0.192	10	HPRT	1.208	0.075	10
RPS7	0.588	0.099	4	RPS7	0.449	0.041	1
YWHAZ	0.547	0.095	3	YWHAZ	0.804	0.055	3
18S	1.199	0.169	9	18S	1.143	0.072	8

Adrenal gland

Gene name	Stability value	Standard error	Ranking
ABL	0.273	0.073	3
ACTB	0.305	0.079	5
GAPDH	0.354	0.088	6
B2M	0.296	0.077	4
GUSB	0.504	0.118	10
HMBS	0.435	0.104	8
HPRT	0.147	0.056	1
RPS7	0.243	0.068	2
YWHAZ	0.452	0.107	9
18S	0.365	0.090	7

Pancreas

Gene name	Stability value	Standard error	Ranking
ABL	1.190	0.265	7
ACTB	2.025	0.423	10
GAPDH	1.125	0.253	6
B2M	0.566	0.166	5
GUSB	0.306	0.158	2
HMBS	0.541	0.164	4
HPRT	1.520	0.326	9
RPS7	0.200	0.188	1
YWHAZ	0.384	0.154	3
18S	1.321	0.289	8

Parathyroid

Gene name	Stability value	Standard error	Ranking
ABL	0.470	0.159	6
ACTB	0.065	0.203	1
GAPDH	0.327	0.130	3
B2M	0.627	0.198	7
GUSB	0.692	0.214	8
HMBS	0.441	0.153	4
HPRT	1.062	0.314	10
RPS7	0.159	0.121	2
YWHAZ	0.469	0.159	5
18S	0.749	0.230	9

Thyroid

Gene name	Stability value	Standard error	Ranking
ABL	0.701	0.187	7
ACTB	0.213	0.127	2
GAPDH	1.230	0.300	9
B2M	0.378	0.132	5
GUSB	0.205	0.128	1
HMBS	0.358	0.130	4
HPRT	1.250	0.304	10
RPS7	0.487	0.147	6
YWHAZ	0.271	0.124	3
18S	1.215	0.297	8

Bone marrow

Gene name	Stability value	Standard error	Ranking
ABL	0.456	0.152	3
ACTB	0.649	0.180	6
GAPDH	1.512	0.349	10
B2M	0.857	0.217	7
GUSB	0.558	0.165	4
HMBS	0.388	0.146	2
HPRT	1.476	0.342	9
RPS7	0.320	0.144	1
YWHAZ	0.620	0.175	5
18S	1.322	0.310	8

Mesenteric lymph node

Gene name	Stability value	Standard error	Ranking
ABL	0.370	0.108	6
ACTB	0.222	0.087	2
GAPDH	0.484	0.129	8
B2M	0.303	0.097	3
GUSB	0.194	0.086	1
HMBS	0.326	0.100	4
HPRT	1.035	0.249	10
RPS7	0.431	0.119	7
YWHAZ	0.328	0.101	5
18S	0.852	0.208	9

Spleen

Gene name	Stability value	Standard error	Ranking
ABL	0.447	0.134	4
ACTB	0.384	0.125	3
GAPDH	0.901	0.225	9
B2M	0.453	0.135	5
GUSB	0.159	0.122	1
HMBS	0.581	0.158	6
HPRT	0.855	0.215	8
RPS7	0.255	0.112	2
YWHAZ	0.629	0.168	7
18S	1.316	0.317	10

Parotid gland

Gene name	Stability value	Standard error	Ranking
ABL	0.141	0.155	1
ACTB	0.248	0.130	2
GAPDH	0.871	0.235	8
B2M	0.535	0.164	6
GUSB	1.040	0.273	9
HMBS	0.428	0.146	5
HPRT	0.838	0.227	7
RPS7	0.261	0.130	3
YWHAZ	0.349	0.135	4
18S	1.518	0.386	10

Duodenum				**Ileum**			
Gene name	Stability value	Standard error	Ranking	Gene name	Stability value	Standard error	Ranking
ABL	0.775	0.218	6	ABL	0.232	0.119	2
ACTB	0.513	0.176	3	ACTB	0.351	0.125	4
GAPDH	0.413	0.167	2	GAPDH	0.597	0.164	5
B2M	1.584	0.387	9	B2M	1.015	0.251	9
GUSB	1.143	0.291	8	GUSB	0.614	0.168	6
HMBS	0.609	0.190	4	HMBS	0.093	0.186	1
HPRT	1.790	0.433	10	HPRT	1.002	0.248	8
RPS7	0.074	0.415	1	RPS7	0.827	0.211	7
YWHAZ	0.727	0.209	5	YWHAZ	0.331	0.123	3
18S	1.022	0.266	7	18S	1.192	0.290	10
Liver				**Kidney**			
Gene name	Stability value	Standard error	Ranking	Gene name	Stability value	Standard error	Ranking
ABL	0.453	0.131	7	ABL	0.279	0.088	3
ACTB	0.572	0.156	8	ACTB	0.310	0.091	4
GAPDH	0.283	0.101	2	GAPDH	0.537	0.122	7
B2M	0.682	0.181	9	B2M	0.715	0.152	8
GUSB	0.282	0.100	1	GUSB	0.452	0.109	6
HMBS	0.337	0.109	5	HMBS	0.368	0.097	5
HPRT	0.315	0.105	4	HPRT	0.830	0.172	9
RPS7	0.289	0.102	3	RPS7	0.222	0.085	2
YWHAZ	0.402	0.121	6	YWHAZ	0.081	0.129	1
18S	1.110	0.282	10	18S	1.391	0.276	10
Myocardium				**Brain**			
Gene name	Stability value	Standard error	Ranking	Gene name	Stability value	Standard error	Ranking
ABL	0.393	0.111	6	ABL	0.239	0.069	2
ACTB	0.105	0.094	1	ACTB	0.151	0.062	1
GAPDH	0.193	0.083	3	GAPDH	0.313	0.079	5
B2M	1.108	0.265	10	B2M	0.511	0.113	8
GUSB	0.435	0.118	7	GUSB	0.347	0.084	7
HMBS	0.225	0.085	4	HMBS	0.298	0.077	4
HPRT	0.757	0.186	9	HPRT	0.334	0.082	6
RPS7	0.109	0.092	2	RPS7	0.249	0.070	3
YWHAZ	0.265	0.090	5	YWHAZ	0.249	0.070	3
18S	0.533	0.138	8	18S	1.052	0.217	9
Blood				**Neoplastic tissues**			
Gene name	Stability value	Standard error	Ranking	Gene name	Stability value	Standard error	Ranking
ABL	0.354	0.127	2	ABL	0.625	0.145	6
ACTB	0.524	0.156	5	ACTB	0.324	0.094	2
GAPDH	0.368	0.129	3	GAPDH	0.467	0.116	4
B2M	0.390	0.132	4	B2M	0.504	0.122	5
GUSB	0.577	0.167	6	GUSB	0.632	0.146	8
HMBS	1.174	0.302	10	HMBS	0.381	0.102	3
HPRT	0.877	0.233	8	HPRT	0.785	0.176	10
RPS7	0.887	0.235	9	RPS7	0.318	0.093	1
YWHAZ	0.350	0.126	1	YWHAZ	0.696	0.158	9
18S	0.662	0.185	7	18S	0.626	0.145	7

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